Université de Montréal

Rôle du système rénine-angiotensine intrarénal dans l'hypertension et les dommages rénaux chez les souris transgéniques diabétiques

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Rôle du système rénine-angiotensine intrarénal dans l'hypertension et les dommages rénaux chez les souris transgéniques diabétiques

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RÉSUMÉ

Plusieurs expériences et études cliniques ont démontré que l'activation du système rénine-angiotensine (RAS) peut induire l'hypertension, un facteur de risque majeur pour les maladies cardiovasculaires et rénales. L'angiotensinogène (Agt) est l'unique substrat du RAS. Cependant, il n'a pas encore été démontré si l'activation du RAS intrarénal peut à elle seule induire des dommages rénaux, indépendamment de l'hypertension systémique, et ainsi jouer un rôle prépondérant dans la progression de la néphropathie diabétique.

Afin d'explorer le rôle du RAS intrarénal dans les dommages rénaux, un diabète a été induit par l'injection de streptozotocin chez des souris transgéniques (Tg) surexprimant l'Agt de rat dans les cellules des tubules proximaux du rein (RPTC). Les souris Tg diabétiques ont été traitées soit avec des inhibiteurs du RAS (perindopril et losartan), de l'insuline ou une combinaison des deux pour 4 semaines avant d'être euthanasiées. Pour une autre étude, des souris Tg non-diabétiques ont été traitées soit avec des inhibiteurs du RAS, l'hydralazine (vasodilatateur) ou l'apocynine (inhibiteur de la NADPH oxydase) pour une période de 8 semaines avant l'euthanasie. Des souris non-Tg ont été utilisées comme contrôles. Des cellules immortalisées de tubule proximal de rat (IRPTC) transfectées de manière stable avec un plasmide contenant l'Agt ou un plasmide contrôle ont été employées comme modèle in vitro.

Nos résultats ont démontré que les souris Tg présentaient une augmentation significative de la pression systolique, l'albuminurie, l'apoptose des RPTC et l'expression de gènes pro-apoptotiques par rapport aux souris non-Tg. Les mêmes changements ont été observés chez les souris Tg diabétiques par rapport aux souris non-Tg diabétiques. L'insuline et/ou les inhibiteurs du RAS ont permis d'atténuer ces changements, sauf l'hypertension qui n'était réduite que par les inhibiteurs du RAS. Chez les IRPTC transfectées

avec l'Agt in vitro, les hautes concentrations de glucose augmentent l'apoptose et l'activité de la caspase-3 par rapport aux cellules contrôles et l'insuline et/ou les inhibiteurs du RAS empêchent ces augmentations.

En plus des changements physiologiques, les RPTC des souris Tg présentent aussi une augmentation significative de la production des espèces réactive de l'oxygène (ROS) et de l'activité de la NADPH oxydase, ainsi qu'une augmentation de l'expression du facteur de croissance transformant-beta 1 (TGF-β1), de l'inhibiteur activateur du plasminogène de type 1 (PAI-1), des protéines de la matrice extracellulaire, du collagène de type IV et de la sous-unité p47 de la NADPH oxydase. Le traitement des souris Tg avec l'apocynine et le perindopril a permis d'améliorer tous ces changements, sauf l'hypertension qui n'était pas corrigée par l'apocynine. D'autre part, l'hydralazine a prévenu l'hypertension, sans modifier l'albuminurie, l'apoptose des RPTC ou l'expression des gènes pro-apoptotiques.

Ces résultats montrent bien que l'activation du RAS intrarénal et l'hyperglycémie agissent de concert pour induire l'albuminurie et l'apoptose des RPTC, indépendamment de l'hypertension systémique. La génération des ROS via l'activation de la NADPH oxydase induit en partie l'action du RAS intrarénal sur l'apoptose des RPTC, la fibrose tubulo-interstitielle et l'albuminurie chez les souris Tg.

D'autre part, une expérience en cours a tenté d'encore mieux délimiter les effets de l'activation du RAS intrarénal, tout en éliminant la néphrotoxicité du STZ. Pour cette étude, les souris Tg surexprimant l'Agt de rat dans leurs RPTC ont été croisées aux souris *Ins2*^{Akita}, un modèle spontané de diabète de type I, afin de générer des souris Akita-rAgt-Tg. Les résultats préliminaires indiquent que le RAS intrarénal est activé dans les souris Akita et que la combinaison avec l'hyperglycémie induit du stress du réticulum endoplasmique (ER) dans les RPTC *in vivo*. Le stress du ER contribue à l'apoptose des RPTC observée dans le diabète, à tout le moins dans le modèle Akita. Le traitement avec des inhibiteurs du RAS permet d'atténuer certains des dommanges rénaux observés dans les souris Akita-rAgt-Tg.

Mots-clés: Rein, système rénine-angiotensine, néphropathie diabétique, hypertension, apoptose, fibrose tubulo-interstitielle, ROS, NADPH oxidase, stress du ER, modèle de souris *Ins2*^{Akita}

ABSTRACT

Experimental and clinical studies have shown that renin-angiotensin system (RAS) activation may lead to hypertension, a major cardiovascular and renal risk factor. Angiotensinogen (Agt) is the sole substrate of the RAS. However, it is unclear whether intrarenal RAS activation alone could induce kidney injury independently of systemic hypertension and play an important role in the progression of diabetic nephropathy (DN). To explore the role of intrarenal RAS in kidney injury, transgenic (Tg) mice overexpressing rat Agt in their renal proximal tubular cells (RPTCs) were rendered diabetic by streptozotocin (STZ). Diabetic Tg mice were treated with RAS blockers (perindopril and losartan), insulin or a combination of both and then euthanized after 4 weeks of treatment. In a separate study, non-diabetic Tg mice were treated with RAS blockers or hydralazine (a vasodilator) or apocynin (an NADPH oxidase inhibitor) and then euthanized after 8 weeks of treatment. Non-Tg littermates served as controls in both studies. Immortalized rat proximal tubule cells (IRPTCs) stably transfected with Agt cDNA or control plasmid were used in the experiments as an in vitro model.

Our results showed that non-diabetic Tg mice displayed a significant increase in systolic blood pressure (SBP), albuminuria, RPTC apoptosis, and proapoptotic gene expression. Diabetic Tg mice had a further increase of albuminuria, RPTC apoptosis, and proapoptotic gene expression, though the SBP of the diabetic Tg mice was similar to that of non-diabetic Tg mice. RAS blockers and/or insulin treatments markedly attenuated these changes, except that insulin had no impact on hypertension. In vitro, high-glucose melieu significantly increased apoptosis and caspase-3 activity in Agt stable transfectants compared to control cells, and these changes were attenuated by insulin and/or RAS blockers.

Furthermore, non-diabetic Tg mice showed significantly elevated reactive oxygen species (ROS) production and NADPH oxidase activity, as well as

enhanced expression of transforming growth factor-beta 1 (TGF-β1), plasminogen activator inhibitor-1 (PAI-1), extracellular matrix proteins, collagen type IV, and NADPH oxidase subunit p47 in their RPTC. Treatment with apocynin and perindopril ameliorated these changes, but apocynin had no effect on SBP. In contrast, hydralazine prevented hypertension but not albuminuria, RPTC apoptosis, or proapoptotic gene expression.

These data indicate that intrarenal RAS activation and hyperglycemia act in concert to induce albuminuria and RPTC apoptosis independent of systemic hypertension. ROS generation via NADPH oxidase activation mediates, at least in part, intrarenal RAS action on RPTC apoptosis, tubulointerstitial fibrosis and albuminuria in Tg mice.

On the other hand, in an on-going experiment, to avoid the nephro-toxic effects of STZ and further delineate the effects of intrarenal RAS activation, Tg mice overexpressing rat Agt in their RPTCs were crossbred with *Ins2*^{Akita} mice, a spontaneous type I diabetes model, to generate Akita-rAgt-Tg mice. Preliminary data indicated that hyperglycaemia and intrarenal RAS activation induced endoplasmic reticulum (ER) stress in RPTC *in vivo,* and the ER stress pathway contributed to RPTC apoptosis in diabetes, at least in the Akita model. RAS blockade was effective in attenuating some parameters of renal injury in Akita-rAgt-Tg mice.

Key words: Kidney, renin-angiotensin system, diabetic nephropathy, hypertension, apoptosis, tubulointerstitial fibrosis, ROS, NADPH oxidase
ER stress, *Ins2*^{Akita} mouse model

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List of Abbreviations

α-SMA: α-smooth muscle actin

8-OHdG: 8-hydroy-deoxyguanosine

ACE: angiotensin converting enzyme

ADA: American Diabetes Association

ADH:antidiuretic hormone

AGEs: advanced glycation end-products

Agt: Angiotensinogen

AIF: apoptosis-inducing factor

Ang: angiotensin

AR : aldose reductase

ARBs: angiotensin receptor blockers

ASK1: Apoptosis signal-regulating kinase 1

AT1R: angiotensin II subtype I receptor

AT2R: angiotensin II sybtype 2 receptor

ATF: activating transcription factor

BENEDICT: Bergamo Nephrologic Diabetes Complications Trial

BIK: Bcl2-interacting killer

BiP: binding immunoglobulin protein

BP: blood pressure

CAD: Caspase-Activated DNAse

CDA: Canadian Diabetes Association

CHOP: ((C/EBP (CCAAT/enhancer-binding protein) homologus protein

CIHI: Canadian Institute for Health Information

CKD: chronic kidney disease

CML: carboxymethyl

CNS: central nervous system

CTGF: connective tissue growth factor

DAG: diacylglycerol

DED: Death Effector Domain

DFF/CAD: DNA-Fragmentation Factor/ Caspase-Activated DNAse

DIABLO: direct inhibitor of apoptosis (IAP)-binding protein with low pI

DISC: Death Inducing Signaling Complex

DM: Diabetes mellitus

DN: Diabetic nephropathy

DR5: death receptor 5

ECM: extracellular matrix

ELISA: Enzyme-Linked ImmunoSorbent Assay

EMT: epithelial-mesenchymal transition

Endo G: Endonuclease-G

ER: endoplasmic reticulum

ERAD: ER-associated degradation

ERK: extracellular signal-regulated kinase

ERO1: endoplasmic reticulum oxidoreductin 1

ESRD: end–stage renal disease

FADD: Fas-Associated via Death Domain

FasL: Fas ligand

GADD: growth arrest and DNA damage

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GBM: glomerular basement membrane

GFAT: glutamine: fructose-6 phosphate amidotransferase

GFR: glomerular filtration rate

GlcNAc: *N*-acetyl glucosamine

GSH: reduced glutathione

HE: hematoxylin-eosin

IAPs: Inhibitor of Apoptosis Proteins

ICAM-1: intercellular adhesion molecule-1

IRE1: inositol-requiring enzyme 1

IRPTCs: immortalized renal proximal tubular cells

JAK/STAT: Janus kinase/signal transducers and activation of transcription

JG apparatus: Juxtaglomerular apparatus

JNK/SAPK: c-Jun N-terminal kinase/stress-activated protein kinase

KAP: kidney androgen-regulated protein

KO: knockout

MAPKs: mitogen-activated protein kinases

MCP-1: monocyte chemoattractant protein-1

MMPs: matrix metalloproteinases

MPO: myeloperoxidase

NADPH: nicotinamide-adenine dinucleotide phosphate

NEP: neutral endopeptidase

NF-κB: nuclear factore-κB

NIDDK: National Institute of Diabetes and Digestive and Kidney Diseases

NKF: National Kidney Foundation

NO: nitric oxide

NOD: nonobese diabetic

OK: opossum kidney

OMI/HTRA2: High Temperature Requirement Protein-A2

$ONOO^-$: peroxynitrite anion

PA: plasminogen activator

PAI-1: plasminogen activator inhibitor-1

PARP: poly (ADP-ribose) polymerase

PERK: pancreatic ER kinase (PKR)-like ER kinase

PKC: protein kinase C

PP1: protein phosphatase 1

PPARs: Peroxisome proliferator-activated receptors

PS: phosphatidyl serine

PTCs: proximal tubular cells

PTs: proximal tubules

RAGE: receptor for AGEs

RAP: receptor-associated protein

RAS: renin-angiotensin system

RIA: radioimmunoassay

ROS: Reactive Oxygen Species

RPT: renal proximal tubules

RPTC: renal proximal tubular cells

RRT: renal replacement therapy

SBP: systolic blood pressure

SD rats: Sprague Dawley rats

SHR: spontaneous hypertension rat

SMAC: Second Mitochondria-Derived Activator of Caspase

SOD: superoxide dismutase

STZ: streptozotocin

T1D: type 1 diabetes

T2D: type 2 diabetes

TEF: transcription enhancer factor

Tg: transgenic

TGF-β1: transforming growth factor-β1

TNFR1: Tumor Necrosis Factor Receptor-1

TRADD: Tumor Necrosis Factor Receptor-1-Associated Death Domain

TRAF2: TNF receptor associated factor 2

TUNEL: Terminal deoxynucleotidyl Transferase dUTP nick end labeling

TZD: thiazolidinediones

UDP: uridine diphosphate

UPR: unfolded protein response

VEGF: vascular endothelial growth factor

VSMCs: vascular smooth muscle cells

XBP-1: X-box binding protein 1

XIAP: X-chromosome-linked inhibitor of apoptosis protein

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Chapter 1: Introduction

1.1 Renal physiology and histology

1.1.1 Renal physiology

The kidney is the most important organ for maintaining homeostasis of the organism regarding both its volume and composition. It is responsible for the removal of metabolic waste products, such as urea, uric acid, creatinine and other end-products of normal metabolism from the blood, and of foreign substances, such as drugs. The kidney is also essential for regulating extracellular fluid osmolarity and for concentrating electrolytes. Another one of its functions is the secretion of hormones: erythropoietin, renin, etc. Urine is formed by the kidney in 3 steps, beginning with the filtration of protein-free plasma into Bowman's capsule, followed by tubular reabsorption and/or tubular secretion.

The nephron, the basic functional unit of the kidney, consists of a renal tubule and a renal corpuscle (a glomerulus enclosed in a Bowman's capsule) (Figure 1-1) [1]. Urine is formed by the nephron-filtration and collecting duct systems. There are approximately 1.7 to 2.4 million nephrons in both kidneys. The typical glomerular filtration rate (GFR) is around 180 litres per day, or 125 ml per minute. However, only 1-2 litres of urine are excreted per day, implying that 99% of the filtered volume is reabsorbed.

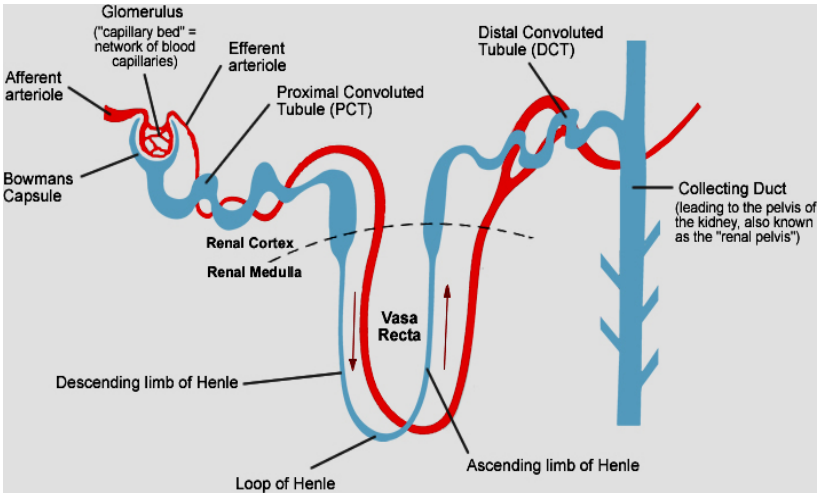

Figure 1-1. Simple diagram of a kidney nephron (ref.[1])

1.1.2 Renal histology

Light microscopy reveals 2 general classes of nephrons, based on the location of the renal corpuscles: cortical nephrons, whose renal corpuscles are in the superficial renal cortex; and juxtamedullary nephrons, whose renal corpuscles are located near the renal medulla. A renal corpuscle is the beginning of a nephron. It is composed of a glomerulus and Bowmans's capsule (or "Malphigian corpuscle").

The glomerulus is a capillary tuft. It receives its blood supply from an afferent arteriole of the renal circulation. Water and solutes from the blood filter into Bowman's capsule by glomerular blood pressure (BP), resulting in ultrafiltrate formation. About 20% of plasma passing through the kidneys filters into Bowman's capsule. The remainder of the blood passes into efferent arterioles, which are narrower than afferent arterioles, forming glomerular BP.

1.1.2.1 Renal filtration barrier

The ultrafiltrate passes through the endothelial cells, the glomerular basement membrane (GBM), and podocytes, which form the renal filtration barrier. The ultrafiltrate then enters the proximal tubules (PTs). However, endothelial cells lining the glomerulus are not usually regarded as part of the renal filtration barrier because they exhibit such large openings that any substance smaller than an erythrocyte can pass through.

The glomerular endothelium sits on the GBM, which is about 250-350 nm thick. The GBM contains 3 layers: the lamina rara externa − adjacent to podocyte processes, the lamina densa − a dark central zone, and the lamina rara interna − adjacent to the endothelial cells. The GBM is rich in negatively-charged glycosaminoglycans, such as heparan sulfate, which serves as part of a charge-selectivity barrier. The negatively-charged basement membrane repels negatively-charged proteins from the blood, preventing proteins from passing into Bowman's space.

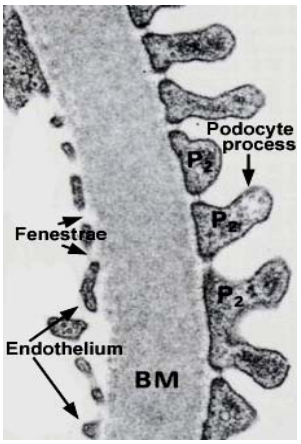

Figure 1-2. Renal filtration barrier (ref. [2])

On the other side of the GBM are the podocytes, which cover the GBM with interdigitating foot processes (or “pedicels”). The space between adjacent podocyte foot processes is called the filtration slit, spanned by a slit diaphragm, which is composed of a number of cell-surface proteins, including nephrin, podocalyxin, etc., ensuring that large macromolecules, such as serum albumin and γ-globulin, remain in the bloodstream. Figure 1-2 illustrates the renal filtration barrier [2].

1.1.2.2 Other components of nephrons

(a)Intraglomerular mesangial cells

Intraglomerular mesangial cells are located among mesangial matrices within a renal corpuscle. They regulate blood flow of the glomerular capillaries by their contractile activity and contribute to the formation of extracellular matrix (ECM), including fibronectin and type IV collagen. Phagocytosis is another function of the intraglomerular mesangial cells; GBM components and immunoglobins can be phagocytosed by mesangial cells.

(b) Renal tubules

Another portion of the nephron is the renal tubule, which contains a PT, Henle’s loop and a distal convoluted tubule. Functionally, the most important segment, the PT can be divided into 2 parts: the proximal convoluted tubule (Figure 1-3 A) and the proximal straight tubule. The most distinctive characteristic of the PT is its brush border, i.e. microvilli on the luminal surface.

They increase the luminal surface area of proximal tubular cells (PTCs) to facilitate their resorptive function. One hundred percent of glucose and amino acids as well as most water, salt and urea are reabsorbed by PTs from the ultrafiltrate.

In detail, glucose is reabsorbed by a fixed number of carrier molecules on the membranes of proximal convoluted tubules. The ability to reabsorb glucose from the filtrate depends on its level in the blood. For example, in the diabetic condition, the glucose level in blood is so high that the number of carrier molecules is not sufficient to transport all of it in the filtrate; therefore, glucose remains in the urine, and glycosuria develops. Normally, by the end of the PTs, all filtered glucose should be reabsorbed. Another function of PTCs is secretion: they secrete hydrogen ions, creatinine, etc. into the lumen (Figure 1-3 B).

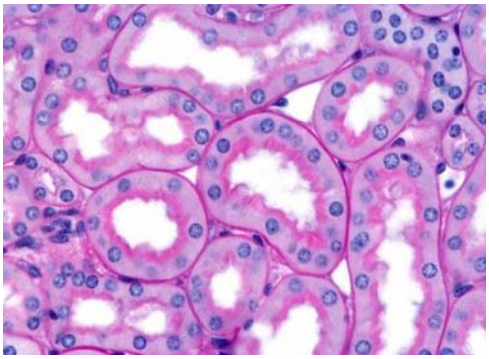

Figure 1-3A. Proximal convoluted tubules (ref. [3])

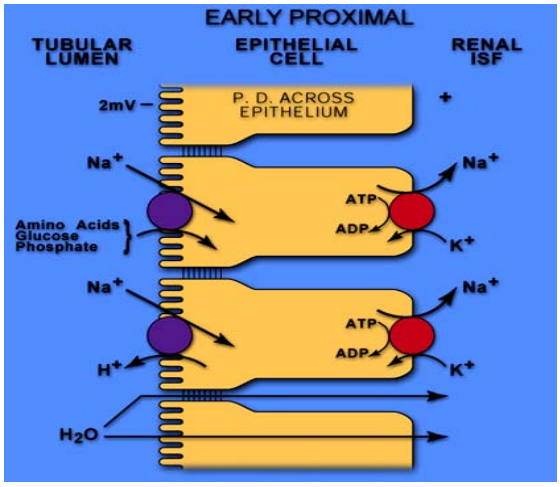

Figure 1-3B. Reabsorption of glucose and amino acids by proximal convoluted tubules (ref.[4])

(c) Henle's loop

After the PT comes Henle's loop (Figures 1-1 and 1-4). It can be divided into 4 parts: the descending limb of Henle's loop, the thin ascending limb of Henle's loop, the medullary thick ascending limb of Henle's loop, and the cortical thick ascending limb. One special effect of Henle's loop is to establish medullary hyperosmolarity. It transports NaCl from the tubule lumen to the interstitium with almost no water, building an hyperosmotic medullary interstitium and delivering hyposmotic tubule fluid to the distal tubule.

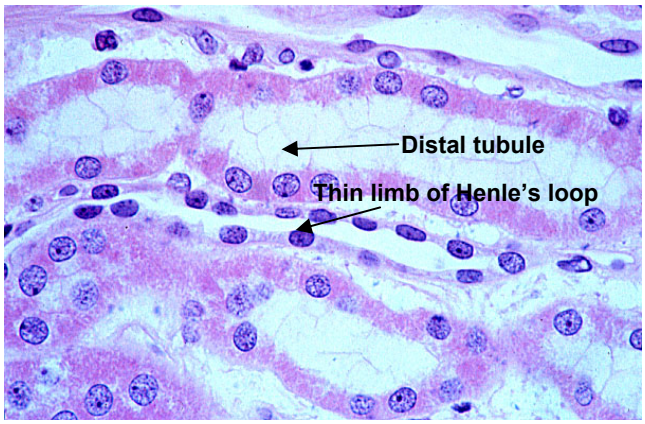

Figure 1-4. Thin limb of Henle's loop and distal tubule (ref. [5])

(d) Distal tubules

The distal convoluted tubule is the final segment of the nephron. Distal tubular cells are simple cuboidal cells that are shorter than PTCs. They reabsorb H_2O and sodium, and secrete potassium, protons, etc. Aldosterone can increase sodium reabsorption by the distal tubule. After the distal tubule, the collecting duct connects the nephrons to the ureter. It is the last component of the kidney to influence the body's electrolyte and fluid balance.

(e) Juxtaglomerular (JG) apparatus

The JG apparatus (Figure 1-5) [6] [7] is a unique segment of the nephron located in the renal cortex, where the afferent arteriole and distal convoluted tubule make direct contact with each other. The JG apparatus works to regulate filtrate formation and systemic BP. It is composed of JG cells, macula densa cells and extraglomerular mesangial cells (or polar cushion cells). JG cells can sense decreases in systemic BP and secrete renin to activate the renin-

angiotensin system (RAS), leading to increased BP. The macula densa cells function as chemoreceptors; they sense changes in solute concentration and flow rate of the filtrate. When sodium concentration in the filtrate gets low, macula densa cells stimulate JG cells to release renin to retain more sodium and expel more potassium. The function of the extraglomerular mesangial cells, however, remains somewhat unknown.

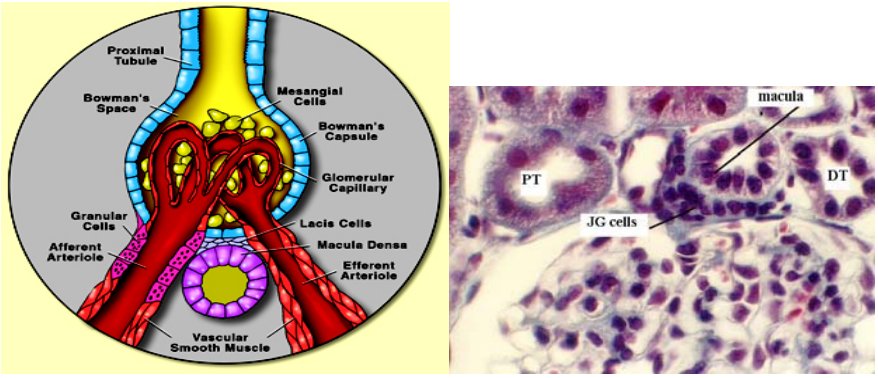

Figure 1-5. The JG apparatus (refs.[6], [7])

1.2 Pathological changes in kidney diseases

In the early stages of kidney disease, histological changes can vary because of many different causes. However, the pathological alterations of the kidneys at end-stage are similar: most glomeruli become sclerotic, and the affiliated tubular cells turn atrophic. Other commonly-observed characteristics are tubules with dilated lumens, tubulointerstitial fibrosis, scattered chronic inflammatory cell infiltrates, and tubules filled with pink casts (Figure 1-6) [8, 9]. Hypertension and renal ischaemia also affect the arteries in the kidneys, making them thick and narrowing their lumen.

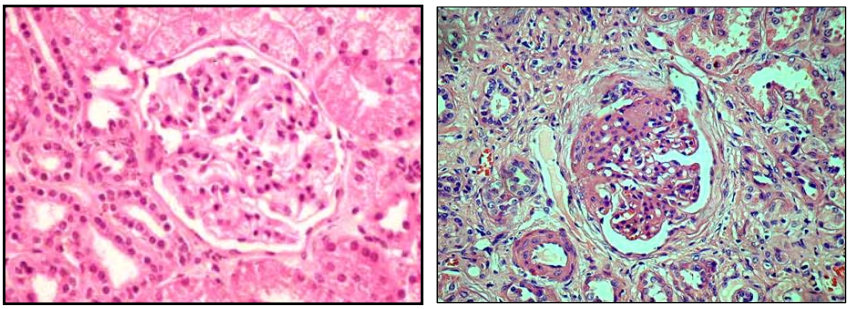

Figure 1-6. A: Normal renal histology, B: End stage renal disease (ESRD) (refs. [8], [9])

Grossly, diseased kidneys are pale, small and bilaterally hard; cross-sectionally, the cortex is thinner. The boundary of the cortex and medulla is no longer as clear as in the normal condition. Additionally, the size of the kidneys in end-stage renal disease (ESRD) is smaller and the surface is granular with more or less scattered, shallow cortical scars (Figure 1-7) [10].

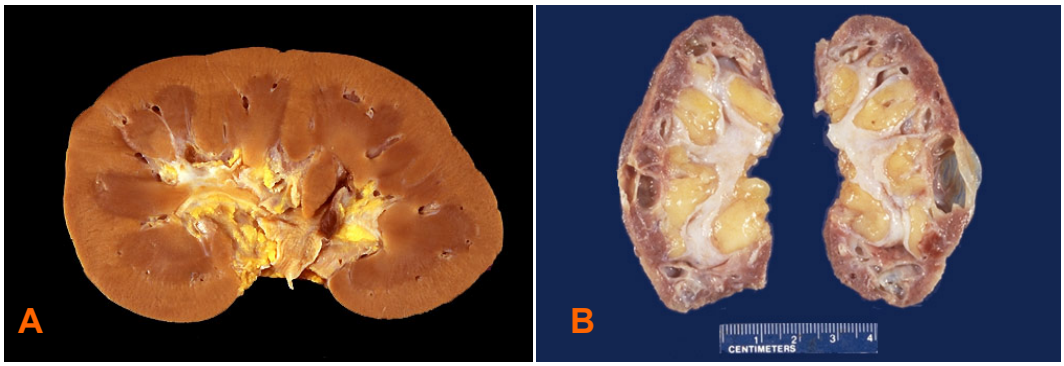

Figure 1-7. A: Normal kidney; B: ESRD (ref. [10])

1.3 Chronic kidney diseases (CKD)

1.3.1 CKD and GFR

Regardless of the cause, kidney damage and decreased function lasting longer than 3 months are called CKD [11], which is a progressive loss of renal function. Analyzing the GFR is the best way to measure the level of kidney function and to determine the stage of kidney disease progression. CKD can be divided into 5 stages based on the GFR, as shown in Table 1-1: the GFR decreases with increasing stage. According to the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) [12], stage 5 CKD is also referred to as ESRD, wherein there is total or near-total loss of kidney function. Patients with stage 5 CKD need dialysis or kidney transplantation to survive. However, since the GFR tends to decline with age, it is necessary to take this into consideration when evaluating kidney function. The average estimated GFR for different age groups, according to the National Kidney Foundation, is given in Table 1-2 [13].

Table 1-1. The 5 stages of chronic kidney disease (CKD) (ref. [12])

Stage	Description	GFR* mL/min/1.73m^2
1	Slight kidney damage with normal or increased filtration	More than 90
2	Mild decrease in kidney function	60–89
3	Moderate decrease in kidney function	30–59
4	Severe decrease in kidney function	15–29
5	Kidney failure requiring dialysis or transplantation	Less than 15

Table 1-2. Average estimated GFR by age (ref. [13])

Age	GFR
20-30	116 ml/min/1.73m^2
30-40	107 ml/min/1.73m^2
40-50	99 ml/min/1.73m^2
50-60	93 ml/min/1.73m^2
60-70	85 ml/min/1.73m^2
70$^+$	75ml/min/1.73m^2

1.3.2 Incidence, prevalence and causes of ESRD

Incidence is defined as the number of newly-diagnosed patients in a specific population during a given time period. Prevalence indicates the number of patients in a specific population at a given point. The incidence and prevalence of ESRD continue to grow worldwide. Globally, at the end of 2005, about 1,900,000 people were receiving renal replacement therapy (RRT), though precise estimates of ESRD incidence and prevalence remain elusive due to incomplete registries of ESRD patients in international databases [14]. In the USA, for example, there were 104,364 new cases of ESRD in 2004 − equivalent to an annual incidence of 342 cases per million, according to the US Renal Data System 2006 annual report [15]. The prevalence of ESRD in the USA in 2003 was 494,471 (1,555 cases per million population). In comparison, based on data pooled from the European Renal Association-European Dialysis and Transplant Association Registry and the UK Renal Registry, the incidence of treated ESRD (based on the incidence of RRT) in 2004 ranged from 75 cases per million in

Iceland to 195 cases per million in Greece. The prevalence of treated ESRD in 2004 ranged from about 479 cases per million in Iceland to 1,022 cases per million in Italy [15]. The NIDDK reported in 2007 that more than 450,000 Americans were undergoing treatment for ESRD in 2003. The major causes of ESRD are diabetes and high blood pressure. According to the American Diabetes Association (ADA), about half of all ESRD patients also have diabetes. The NIDDK asserted that diabetes and hypertension combined account for more than 60% of all ESRD cases, although there are other potential causes of ESRD, including other CKDs, heart failure and lupus. Patients with type 1 diabetes (T1D) may progress to ESRD within 5 to 10 years if they have chronic proteinuria. T1D patients are 12 times more likely to develop ESRD than type 2 diabetes (T2D) patients, according to the ADA. However, T2D is far more common and, therefore, produces more total cases of ESRD [17]. Other possible causes of kidney disease that may lead to ESRD are glomerulonephritis, vasculitis, interstitial nephritis and toxicity, etc. Figure 1-8 summarizes the causes of ESRD [16].

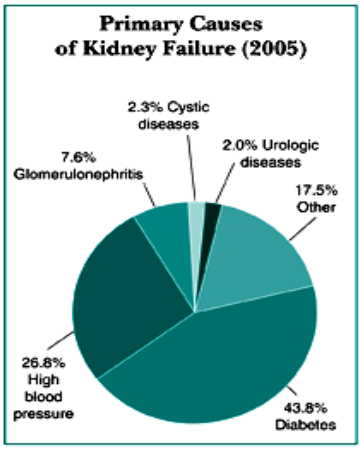

Figure 1-8. Causes of ESRD (ref. [16])

The Canadian Institute for Health Information (CIHI) reported in 2005 that from 1998 to 2002, the prevalence of ESRD in Canada increased 33%, while the incidence rose 17%; the incidence rate of ESRD was 153 per million. During this period, annual kidney transplants performed in Canada increased 2.5%. Expenditures on ESRD were about 1.2% of total health care costs. More precisely, the annual cost per ESRD patient is Cdn $55,466 [17].

1.4 Diabetes mellitus (DM)

1.4.1 Prevalence and cost of diabetes

In 2007, an estimated 246 million people worldwide were affected by diabetes. With a further 7 million people developing diabetes each year, that number is expected to hit 380 million by 2025 [18]. In the USA, 14.6 million people were diagnosed with diabetes in 2005; and it is believed that another 6.2 million diabetic patients were undiagnosed. Therefore, 20.8 million people, or about 7% of the American population, have diabetes[19]. According to the Canadian Diabetes Association (CDA), more than 2 million Canadians have diabetes. By the end of the decade, this number is expected to reach 3 million. Based on CDA data, diabetes and its complications cost the Canadian healthcare system an estimated $13.2 billion per year. By 2010, these costs are expected to rise to $15.6 billion a year and to $19.2 billion a year by 2020 (figure 1-9) [20].

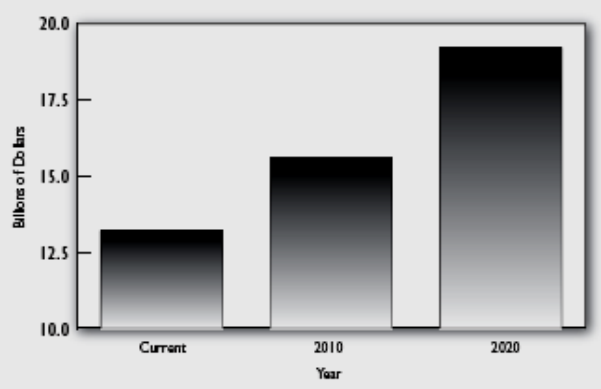

Figure 1-9. Cost of diabetes and its complications in Canada (ref.[20])

1.4.2 Pathogenesis of diabetic complications

Diabetes is characterized by chronic hyperglycaemia and the development of microvascular and macrovascular pathologies. The microvascular complications of diabetes affect small blood vessels, including retinopathy, nephropathy and neuropathy. The central pathological mechanism in macrovascular disease is the process of atherosclerosis, which leads to narrowing of arterial walls throughout the body, evoking coronary heart disease, myocardial infarction, stroke, etc. Numerous researchers are working on the

mechanisms of hyperglycaemia-induced cell injury. Brownlee's group summarized 4 main hypotheses on how hyperglycaemia produces diabetic complications [21]. They are: increased polyol pathway flux, augmented advanced glycation end-product (AGE) formation, activation of protein kinase C (PKC) isoforms; and increased hexosamine pathway flux [21].

(1) Increased polyol pathway flux

The polyol pathway was first described in peripheral nerves in 1966 [22]. In this pathway, aldose reductase (AR) plays a central role. AR normally reduces toxic aldehydes in cells to inactive alcohols. In the diabetic condition, glucose concentrations in cells become very high. AR also reduces glucose to sorbitol, which is later oxidized to fructose. In the process of reducing glucose to sorbitol, AR consumes the cofactor nicotinamide-adenine dinucleotide phosphate (NADPH). NADPH is also essential for regenerating a critical intracellular antioxidant: reduced glutathione (GSH). By reducing the amount of reduced GSH, the polyol pathway increases susceptibility to intracellular oxidative stress. Lee et al. reported previously that decreased GSH levels were found in the lenses of transgenic (Tg) mice that overexpress AR [23]. The indirect biochemical consequences of heightened sorbitol pathway activity include non-enzymatic glycation initiated by fructose, a glycating agent that is 10 times more potent than glucose [24], PKC activation, oxidative and nitrosative stress, and oxidative stress-mediated downstream events, such as activation of mitogen-activated protein kinases (MAPKs) and poly(ADP-ribose) polymerase (PARP) [25]. Two separate groups using different AR inhibitors demonstrated that its suppression in the nerve improved both nerve physiology and fibre density as well as function (Figure 1-10) [21] [26] [27].

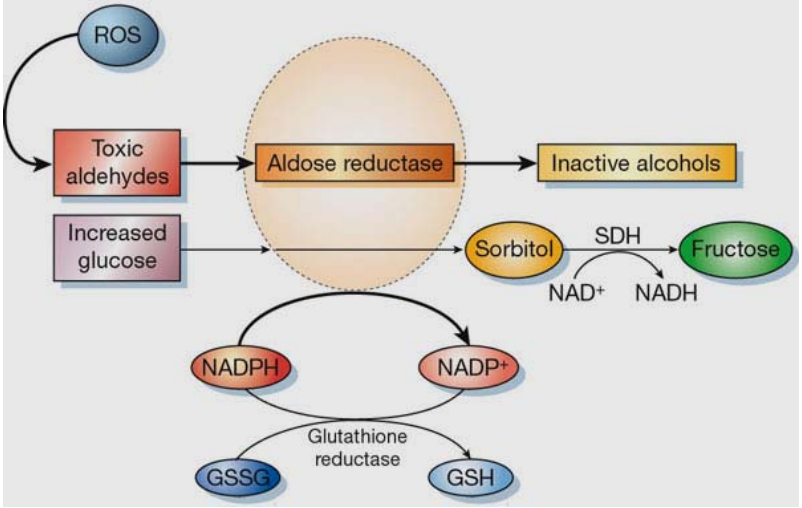

Figure 1-10. Aldose reductase (AR) and the polyol pathway (ref. [21])

(2) Increased intracellular formation of AGEs

Intracellular glucose elevation is the initiating event in the formation of both intracellular and extracellular AGEs [28]. AGEs are normally formed non-enzymatically by the interaction of carbonyl (aldehyde or ketone) groups of reducing sugars, such as glucose, and with lysine and N-terminal amino groups in a variety of proteins, lipids, and nucleic acids, which results in the formation of glycation products via the Maillard reaction. These go through further rearrangements and various reactive intermediate products, or AGE precursors, including the formation of α-dicarbonyls or oxoaldehydes. 3-Deoxyglucosone, glyoxal, and methylglyoxal are examples of α-dicarbonyls. They react with amino groups of intracellular and extracellular proteins to form AGEs, an heterogeneous class of stable and irreversible covalent adducts (Figure 1-11) [29]. Physiologically, AGE formation is part of the ageing process. It is thought that the physiological role of advanced glycation is to identify senescent proteins for degradation. Under high glucose ambience, AGE formation increases due to hyperglycaemia [30]. Among the many potential pathogenic factors responsible for the development of diabetic microvascular disease, the advanced glycation pathway is thought to be a pivotal process in mediating tissue damage. Serum and tissue AGE levels are correlated with the time-averaged concentration of

blood glucose and the severity of diabetic complications, including nephropathy and retinopathy. Glycoxidation products, a subclass of AGEs, such as N-(carboxymethyl) lysine (CML) and pentosidine, require both glycation and oxidation for their formation. In diabetic nephropathy (DN), CML and pentosidine accumulate in the expanded mesangial matrix and nodular lesions co-localizing with malondialdehyde-lysine, a lipoxidation product [31]. Schinzel et al confirmed the role of AGEs in diabetic micro- and macroangiopathy by immunohistochemistry showing that AGEs localized in the vascular wall. Both AGE precursors and AGEs alter the functional properties of some important intracellular molecules, such as proteins involved in gene regulation and extracellular molecules. By changing the signaling between the matrix and the cells, AGE precursors and AGEs elicit cellular dysfunction, disrupting normal cell-matrix contact and preventing the maintenance of tissue integrity [32].

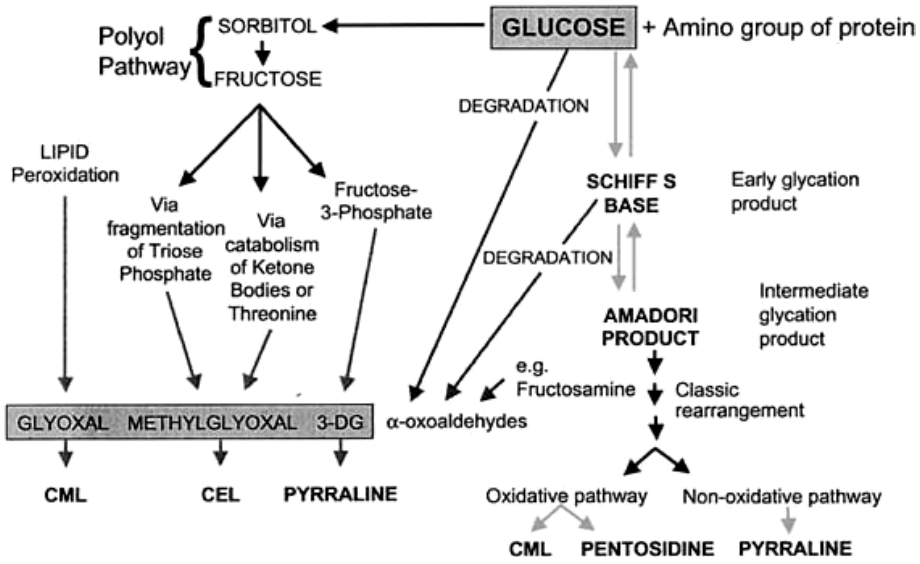

Figure 1-11. Pathways of AGE formation (ref. [29])

Hyperglycaemia may also activate PKC isoforms indirectly via binding to AGE receptors. In a study by Thallas-Bonke et al., where streptozotocin (STZ)-induced diabetic rats were treated with ALT-711, an inhibitor of AGE accumulation, diabetes-evoked increases in PKC-α, -βI, -βII, and -ε isoforms were attenuated in association with decreased renal AGE accumulation. ALT-

711 also reduced the translocation of phosphorylated PKC-α from the cytoplasm to the membrane. ALT-711 treatment suppressed the expression of vascular endothelial growth factor and the ECM proteins fibronectin and laminin, in association with diminished albuminuria. These findings implicate AGEs as important stimuli of PKC activation, particularly PKC-α, in diabetic kidneys [33].

(3) PKC Activation

Among various kinases, PKCs are one of the most extensively studied. PKCs are a family of serine/threonine kinases that comprises 13 isoforms, which can be categorized into 3 groups based on their mode of activation [34]. The group of classical or conventional PKCs (cPKC) consists of the α, βI, βII and γ isoforms, all of which depend on calcium, diacylglycerol (DAG) or its analogue phorbol 12-myristate 13-acetate, and in most cases phosphatidyl serine (PS) for activation. The isoforms that are independent of calcium, but require DAG and PS are classified as the new PKCs (nPKCs), and include the subtypes δ, ε, η, and υ. Isoforms ζ, ι, λ and μ constitute the third group of PKCs, called the atypical PKC (aPKC). Their activation does not require calcium or DAG, only PS. However, PKCs are incapable of activation by DAG and other co-factors unless they are post-translationally or co-translationally phosphorylated. Thus, PKCs are regulated by 2 sequential and equally critical mechanisms: phosphorylation and binding to DAG and/or other co-factors. Each mechanism regulates the structure, subcellular localization and function of PKC [35]. Figure 1-12 is a simple summary of PKC subtypes [36].

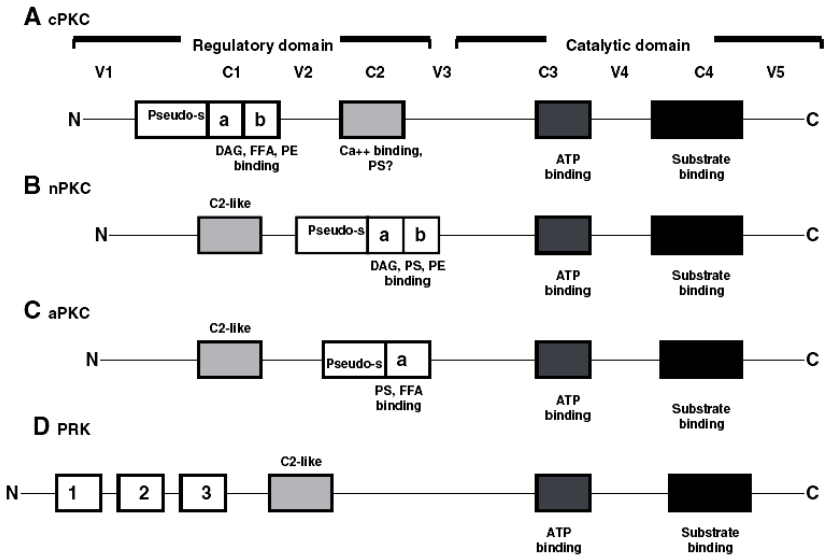

Figure 1-12. Protein architecture and cofactor requirements of various subfamilies of protein kinase C (PKC): all PKC subfamilies, and so all isoforms, contain a regulatory and a catalytic domain. These consist of 4 conserved regions (C1–C4) and 5 variable regions (V1–V5). As can be seen by the differences in protein architecture and cofactor requirements, the inclusion of the PRK (D) as a true subfamily of PKC is controversial. cPKC, conventional PKC; nPKC, new PKC; aPKC, atypical PKC; PRK, protein kinase C regulatory kinase; DAG, diacylglycerol; FFA, free fatty acids; PS, phosphatidyl serine; PE, phorbol ester; pseudo-s, pseudo-substrate; Ca ++ , calcium; ATP, adenosine triphosphate. The cysteine-rich zinc finger regions C1a and C1b are indicated with a and b. (ref. [36])

Many studies have reported that total DAG levels in vascular tissues, such as the retina, heart and renal glomeruli [37] [38] [39] are increased in diabetes. DAG levels can be augmented in the diabetic state through multiple pathways, and subsequently PKC is activated by DAG. Furthermore, the DAG-PKC pathway can be activated by hyperglycaemia-induced elevations of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) which can activate PKC either directly or by increasing DAG production [40, 41]. Multiple diabetic complications have been associated with heightened PKC activation (Figure 1-13) [42]. Increased ECM deposition is a common characteristic of DN, which is due to overexpression of ECM collagen, fibronectin and laminin [43]. High-glucose levels stimulate collagen IV transcription in mesangial cells in vitro. Similarly, PKC agonists stimulate type IV collagen expression [44] and fibronectin accumulation in cultured mesangial cells [45] and PKC inhibitors down-regulated their expression. These results suggest the importance of PKC

activation in the basement membrane remodeling process in the presence of hyperglycaemia.

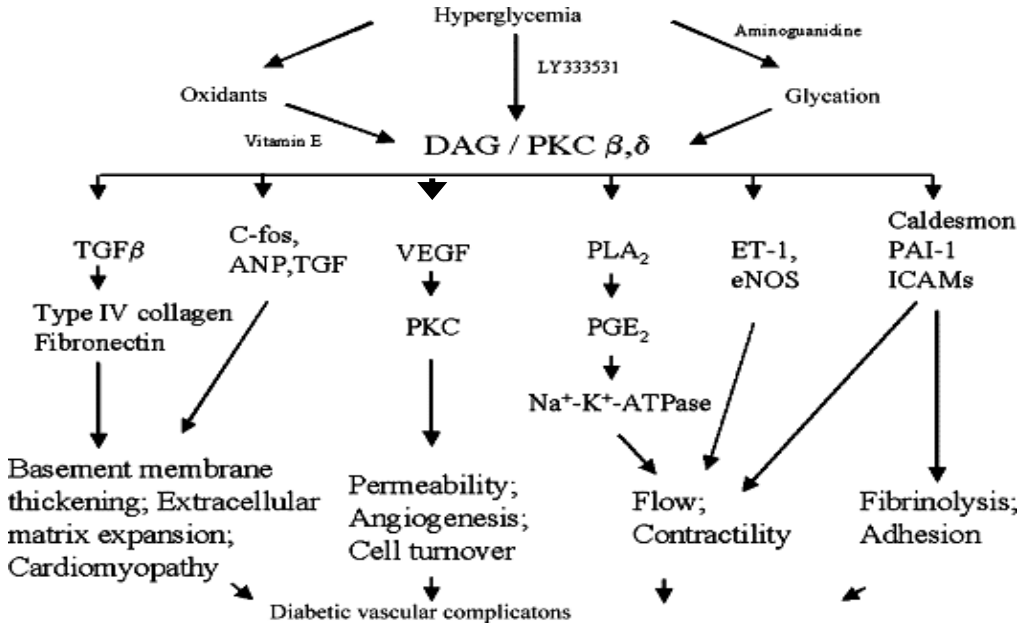

Figure 1-13. Physiological effects and cellular mechanisms of DAG–PKC activation induced hyperglycaemia (ref. [42])

Among various fibrotic factors, transforming growth factor-β1 (TGF-β1) and connective tissue growth factor (CTGF) play key roles in the development of basement membrane thickening and increased ECM in DM [46]. Both CTGF and TGF-β1 can stimulate the production of type IV collagen, fibronectin and laminin in cultured mesangial cells and glomerular epithelial cells[47, 48], contributing to ECM accumulation. It is suggested that PKC activation may be involved in glucose-induced elevation of CTGF and TGF-β1 expression [49]. Therefore, PKC activation in the hyperglycaemic condition could be responsible for heightened levels of matrix protein synthesis mediated either directly or indirectly by stimulating the expression of TGF-β1 and CTGF. Augmented basement membrane levels could partially cause the vascular dysfunction and proteinuria seen in nephropathy. Several studies have demonstrated that PKC activation can directly increase the permeability of macromolecules[50, 51] across the endothelial or epithelial barriers.

To summarize, activated PKC has a variety of effects on gene expression: some genes that are essential for normal function are decreased; whereas others that are detrimental to cells are elevated [52-54].

(4) Hexosamine pathway flux

When intracellular glucose is high, it is metabolized through glycolysis, going first to glucose-6 phosphate, then to fructose-6 phosphate, and enters the glycolytic pathway. However, some fructose-6 phosphate is converted to glucosamine-6 phosphate and finally, to UDP (uridine diphosphate) *N*-acetyl glucosamine by GFAT (glutamine: fructose-6 phosphate amidotransferase). Intracellular glycosylation by the addition of GlcNAc to serine and threonine is catalyzed by the enzyme *O*-GlcNAc transferase. Glycosylated serine and threonine can then bind to transcription factors, such as Sp-1 and augment the synthesis of factors such as TGF-β1 and plasminogen activator inhibitor-1 (PAI-1), both of which are detrimental to blood vessels [55]. The hexosamine pathway generating toxic metabolites is another mechanism by which hyperglycaemia can induce cell damage. However, its role remains to be fully elucidated and the clinical applicability of these findings has been limited by a lack of specific orally-active agents targeting this pathway (Figure 1-14) [21].

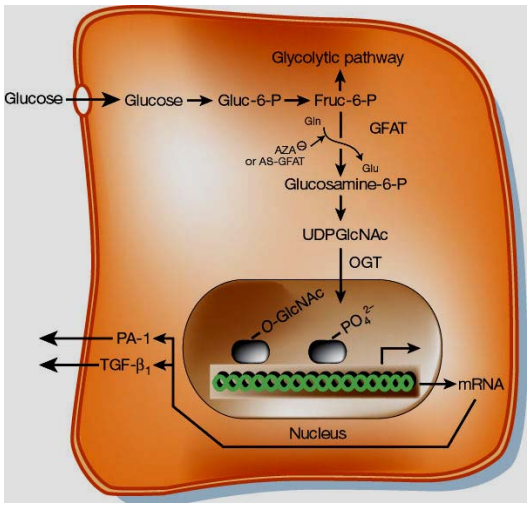

Figure 1-14. The hexosamine pathway (ref. [21])

A unified mechanism

Although data support the above mentioned hypotheses, no apparent common element has linked these mechanisms to each other until several years ago. Moreover, clinical trials inhibiting these pathways in patients were not satisfactory. In recent years, it is hypothesized that all of these mechanisms are linked to a common upstream event and that failure to block all of the downstream pathways could explain the unsatisfactory clinical trials with single-pathway inhibitors. In 2000, 2 separate groups confirmed that the common element linking hyperglycaemia-induced damage is the overproduction of superoxide by the mitent linking these mechanisms to each other until several years ago. Superoxide activates the 4 damaging pathways mentioned by inhibiting glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [41, 55]. Soon after, Du et al. reported that GAPDH was inhibited through the activation of poly(ADP ribose) polymerase [56]. Figure 1-15 demonstrates the 4 mechanisms linked by the common element: superoxide [21].

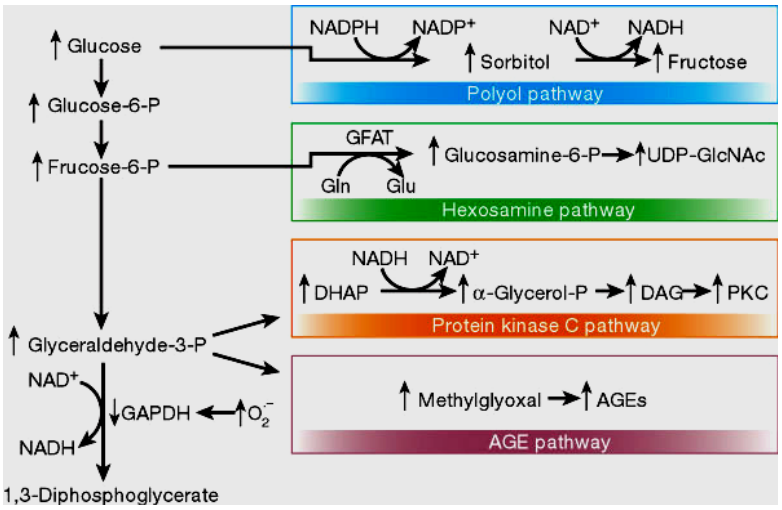

Figure 1-15. Mitochondrial overproduction of superoxide activates 4 major pathways of hyperglycaemic damage by inhibiting GAPDH (ref. [21]).

1.4.3 ROS and oxidative stress

Molecular oxygen (O_2) is essential for all aerobic organisms. Primarily in the mitochondria, aerobic energy metabolism is dependent on oxidative phosphorylation, by which the oxidoreduction energy of mitochondrial electron

transport is converted to the high-energy phosphate bond of ATP. O_2 serves as the final electron acceptor for cytochrome-*c* oxidase which catalyzes O_2 to H_2O. During these electron transfer reactions, partially reduced and highly reactive metabolites of O_2 may be formed. These O_2 metabolites include superoxide anion and H_2O_2. In the presence of transition metal ions, even more reactive hydroxyl radicals may be generated. These partially reduced metabolites of O_2 are often referred to as ROS because of their higher reactivities relative to molecular O_2 [57] (Figure 1-16).

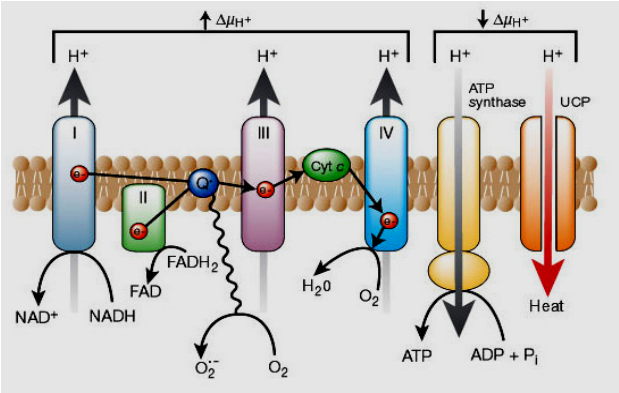

Figure 1-16. Production of superoxide by the mitochondrial electron transport chain (ref.[21])

Under certain conditions, superoxide anion can react with nitric oxide (NO) to produce significant amounts of a much more oxidatively-active molecule, peroxynitrite anion ($ONOO^-$), which is a potent oxidizing agent: $NO^{\bullet} + O_2^{\bullet -} \rightarrow ONOO^-$ [58]. Indeed, all phagocytes have a well-characterized superoxide anion-generating plasma membrane oxidase, which is capable of producing large amounts of ROS required in host defense. ROS generated in nonphagocytic cells have been traditionally regarded as toxic by-products of metabolism because of their potential to damage lipids, to oxidize proteins, and to mutate and cleave DNA [59]. Besides mitochondria, the endoplasmic reticulum (ER), peroxisomes and NADPH oxidase are also sources of ROS production. A variety of cytokines and growth factors that bind receptors of different classes have been reported to generate ROS in nonphagocytic cells as well, though not all functional or pathophysiological effects have been elucidated [57]. Several antioxidant enzymes, such as superoxide dismutase, which reduce

superoxide anion to H_2O_2, and catalase and glutathione peroxidise, which reduce H_2O_2 to H_2O, can protect against the potentially-damaging effects of ROS (figure 1-17) [60]. In addition, non-enzymatic free radical scavengers, vitamin E, vitamin C and glutathione play a critical role in cellular redox balance. Oxidative stress will occur if there is an imbalance between oxidant production and the capacity of the cells to prevent oxidative injury.

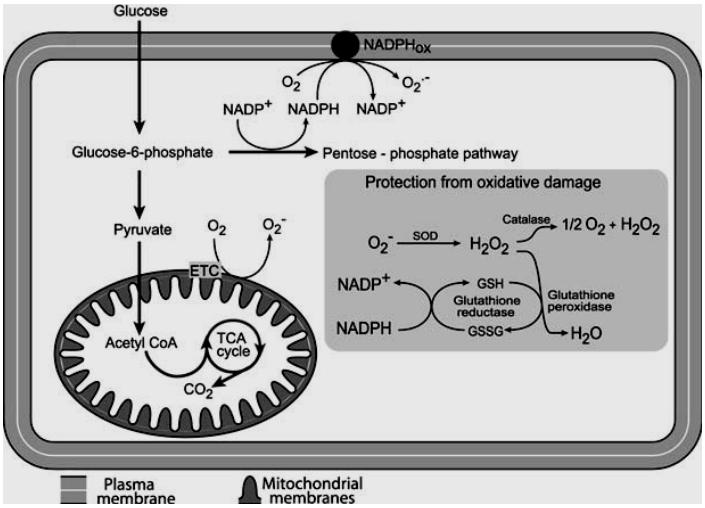

Figure 1-17. Relevant sites of production of ROS and antioxidant systems in a generic cell type (ref.[60])

Oxidative stress is detrimental to cell structures, nucleic acids, lipids and proteins. Hydroxyl radical can react with all components of the DNA molecule, damaging both purine and pyrimidine bases as well as the deoxyribose backbone. 8-OHdG formation is a typical example of a DNA lesion by oxidative stress [61]. Permanent modification of genetic material by ROS could be the first step involved in mutagenesis, carcinogenesis, and other pathological processes, including diabetes. Polyunsaturated fatty acid residues of phospholipids, which are extremely sensitive to oxidation, are another target of ROS generation. 4-Hydroxynonenal, a weak mutagen, appears to be the major toxic product of lipid peroxidation [62]. However, proteins vary significantly in their susceptibility to oxidative damage, for example, intact proteins are less sensitive to oxidation than misfolded proteins. Oxidative modifications of proteins heighten their susceptibility to proteolytic attack. Proteolysis is estimated to increase more than 10-fold after exposure to superoxide radical or H_2O_2 [63]. Based on Stadtman's

finding, the side-chains of all amino acid residues of proteins, particularly cysteine and methionine, are susceptible to oxidation of ROS [64]. Oxidation of cysteine residues may result in the reversible formation of mixed disulphides between protein thiol groups (–SH) and low molecular weight thiols, especially GSH (*S*-glutathiolation). A variety of stress-sensitive intracellular signaling pathways such as nuclear factore-κB (NF-κB), p38 MAPK, c-Jun amino-terminal kinase/ stress-activated protein kinase (JNK/SAPK), hexosamine and others, can be activated, resulting in the increased expression of numerous gene products that may cause cellular damage and thus play a major role in the etiology of diseases [60].

1.5 Diabetic nephropathy (DN)

DN, a common diabetic complication, is a clinical syndrome characterized by progressive renal insufficiency in the setting of hyperglycaemia, persistent albuminuria (microalbuminuria > 30mg/24h, clinical albuminuria > 300mg/24h), hypertension, decreased GFR and a highly-elevated risk of cardiovascular morbidity and mortality [65]. The earliest morphological change of DN is expansion of the mesangial area [66], which is caused by elevated ECM deposition and mesangial cell hypertrophy. GBM thickening is another early histological finding that is already apparent 1 year after the onset of T1D [67]. Moreover, GBM thickening is progressive over years; augmented ECM synthesis and impaired removal contribute to GBM thickening. In the diabetic condition, α1 (IV) and α2 (IV) chains are up-regulated in mesangial cells, whereas α3 (IV) and α4 (IV) expression is increased in the GBM [68]. Deposition of collagen type I and III in the mesangial area occurs late in glomerulosclerosis and is not an early event [69]. In the late stage of DN, glomerulosclerosis, vascular diseases and changes of the tubulointerstitial architecture with tubular atrophy and interstitial fibrosis have all been discerned [66] [70] [71] [72] [73]. Since the original description by Kimmelstiel and Wilson, nodular glomerulosclerosis has been considered as a hallmark of DN [70]. Figure 1-18 shows some typical morphological changes in DN [74] [10].

After 20 years of diabetes duration, around 30% of T1D patients develop DN. Over 5% of newly diagnosed T2D patients already have renal dysfunction. Another 25-40% of T2D patients will develop DN after 25 years of diabetes; they will also manifest a high tendency to progress to ESRD. This difference is based on the fact that T2D can remain undiagnosed for long periods. Several studies have indicated genetic predisposition to DN, and epidemiological investigations confirm that the prevalence of DN depends on ethnic background. Siblings of T1D patients with DN have a higher risk of DN [75]. The relatives of T2D dialysis patients are 5 times more likely to develop ESRD than the relatives of T2D patients without nephropathy [76]. Caucasians of European origin demonstrate the lowest prevalence [77].

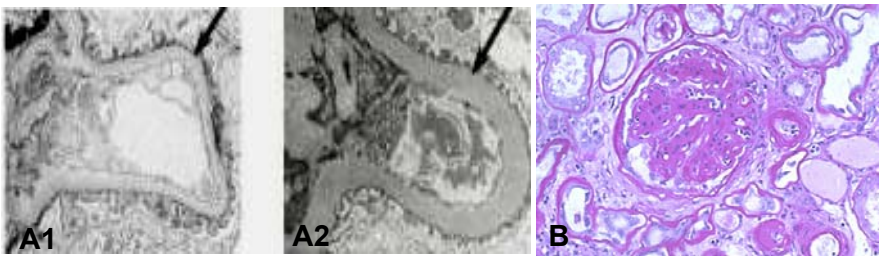

Figure 1-18. A1: Normal GBM, A2: GBM thickening in diabetes
B: Mesangial matrix expansion and sclerosis in a diabetic glomerulus (refs.[74], [10])

1.5.1 Pathogenesis of DN

Although the pathogenesis of DN is not completely understood, several factors are closely relevant to DN. The major factors of hyperglycaemia-induced cell damage have been discussed previously. Briefly, they are: (1) increased formation of AGEs; (2) PKC activation; (3) heightened activity of growth factors; (4) augmented ROS formation; (5) activation of cytokines (e.g. angiotensin II or AngII); (6) enhanced activity of the AR pathway; and (7) decreased glycosaminoglycan content in basement membranes. The relationship of these major factors to the development of DN is discussed below.

1.5.1.1 AGEs and DN

The kidney is a target of AGE-mediated damage. It also contributes to increasing the circulation of AGE concentrations via a decline of renal function,

i.e. the clearance of AGEs [78]. Clinical studies in patients with diabetes have demonstrated that serum levels of AGEs are significantly augmented with progression to microalbuminuria and, subsequently, to overt nephropathy [79]. Makita et al. found an elevation of circulating AGE peptides, which correlated with the severity of renal impairment in both T1D and T2D patients [80]. Tanji et al. reported that DN patients had increased podocyte receptors for AGE (RAGE) expression, and DN severity was correlated with the extent of AGE formation in the glomerular and tubulointerstitial compartments [81]. Animal studies have provided further evidence that AGEs correlate with DN. Diabetic animals manifest significant increases of renal AGEs, as assayed by a range of techniques [82]. Pathological changes in the diabetic kidney are reduced with AGE formation inhibitors or other approaches to diminish AGE accumulation, such as cross-link breakers [83, 84]. Demonstrated by Vlassara et al., AGEs caused kidney damage similar to that seen in DN: elevated renal AGE content and glomerular volume, GBM thickening, mesangial matrix expansion, NF-κB activation, heightened collagen IV and TGF-β mRNA expression [85]. Soulis-Liparota et al. similarly demonstrated the effect of AGEs mediating renal injury in the diabetic condition in animal studies with non-specific AGE formation inhibitors to ameliorate both the structural and functional features of experimental DN without directly influencing glycaemic control [86]. Since then, numerous experiments have supported and extended these findings [87]. The detrimental effects of AGEs on the kidney appear to occur through their interaction with specific RAGEs and binding proteins.

AGEs can interact with multiple pathways, such as inflammatory, oxidative, metabolic, and haemodynamic pathways. These diverse interactions appear to occur mainly through multiligand RAGE, a member of the immunoglobulin superfamily of cell surface molecules [88, 89] and the best characterized signal transduction receptor for AGEs. RAGE is highly expressed by a number of cells whose function is perturbed in diabetes [89]. These receptors are present on various renal cell types, including PTCs, mesangial cells, and podocytes [90] [91] [92] [93]. Numerous studies have implicated

RAGEs in the development and progression of DN [94, 95]. In Tg mice overexpressing the RAGE gene in vascular cells, Yamamoto et al found an accelerated rate of renal damage after the induction of diabetes. The changes they observed included: elevation of albuminuria and serum creatinine levels, mesangial expansion, and advanced glomerulosclerosis [96]. Consistently, diabetic RAGE knockout (KO) mice induced by STZ showed less renal injury compared to diabetic wild-type mice. In particular, these RAGE KO mice did not present significant mesangial expansion or GBM thickening [93]. Table 1-3 is a simple summary of AGE effects on DN.

Table 1-3. Effects of AGEs that potentially contribute to DN (ref. [97])

Extracellular matrix (ECM)
Intermolecular and intramolecular cross-linking
Disruption of cell-matrix and matrix-matrix interactions
Increased ECM synthesis
Tubuloepithelial-mesenchymal transdifferentiation
Reduced metalloprotease activity
Induction of oxidative stress
Increased generation of reactive oxygen species
Activation of NADPH oxidase
Mitochondrial dysfunction
Depletion of endogenous antioxidants
Induction of cytokines and growth factors including
TGF-β1, CTGF, VEGF, IGF-1, PDGF
TNF-α, IL-1B, IL-6
Activation of cellular pathways
PKC-MAPK pathway
Tyrosine kinase pathway
Nuclear factor κB
Other effects
Disruption of vasorelaxation
Genotoxicity

1.5.1.2 PKC and DN

PKC seems to be a centerpiece in the pathogenesis of DN. The importance of PKC isozyme activation in the diabetic milieu initiating early DN was first identified by Ishii et al. [39]. They discovered that when the PKC-β inhibitor LY-333531 was administered orally to STZ-induced diabetic rats, not only did it prevent an elevated GFR, an increased albumin excretion rate, and augmented retinal circulation time, but it also led to the mRNA overexpression of glomerular TGF-β1 and ECM proteins [39] [52]. They observed a similar effect in *db/db* mice, a T2D animal model. LY-333531 also prevented progressive mesangial expansion [98]. Cohen et al. discerned that glycated albumin stimulated mesangial cell PKC-β activity, which is linked to the increased

expression of collagen IV [99]. Stimulation of MAPKs, particularly extracellular signal-regulated kinase (ERK)1/2 after PKC activation, is necessary for mesangial cell growth and enhanced gene expression of growth factors and ECM proteins [44, 100]. In both mesangial cells cultured in high glucose [101, 102] and glomeruli isolated from STZ-induced diabetic rats[103], ERK1/2 activity was significantly higher than in normal glucose controls and was PKC-dependent, although protein expression remained unchanged. It was further determined that high glucose-enhanced mesangial cell TGF-β1 and ECM protein expression was mediated through ERK1/2 [100]. In cultured rat mesangial cells, Ha and Lee found that high glucose generates H_2O_2 within 1 hour and inhibition of PKC blocks high glucose or H_2O_2-induced TGF-β1 and fibronectin mRNA expression and protein synthesis [104]. These data imply that ROS generated under high glucose conditions directly or indirectly stimulates PKC. One mechanism for direct activation of PKC by ROS is through redox changes in sulfhydryl groups on PKC isoform cysteine-rich regions. These redox changes may also cause PKC isozymes to be more responsive to DAG activation during signal transduction[105]. These results suggest that stimulated PKC isozymes have multiple functions in the signaling cascades leading to collagen IV expression and ECM accumulation, and basal PKC-β activities are required for the high-glucose-enhanced mesangial cell expression of ECM protein, contributing to diabetic glomerulosclerosis and tubulointerstitial fibrosis.

1.5.1.3 TGF-β and DN

TGF-β signaling is activated by a large number of mediators that have been identified to induce renal injury in DN. AGEs, ROS, DAG, PKC and AngII are able to activate TGF-β signaling [106] [107]. TGF-β is recognized as the major cytokine responsible for the ECM pathobiology seen in DN and perhaps plays the most crucial role in DN pathogenesis [108]. The TGF-β 1, 2 and 3 isoforms are prototypes of the TGF-β superfamily that exert multiple effects and consist of dimeric proteins with conserved structures. When TGF-β binds to its type II receptor, it trans-phosphorylates type I serine/threonine kinase receptors. The

type I receptor then interacts with Smad2 and 3, which forms a complex with Co-Smad4 [109]. Thirdly, the complex translocates to the nucleus and binds with promoters of TGF-β target genes, e.g., collagen α1, PAI-1, Jun B, c-Jun, and fibronectin and regulates their transcription (figure 1-19) [110].

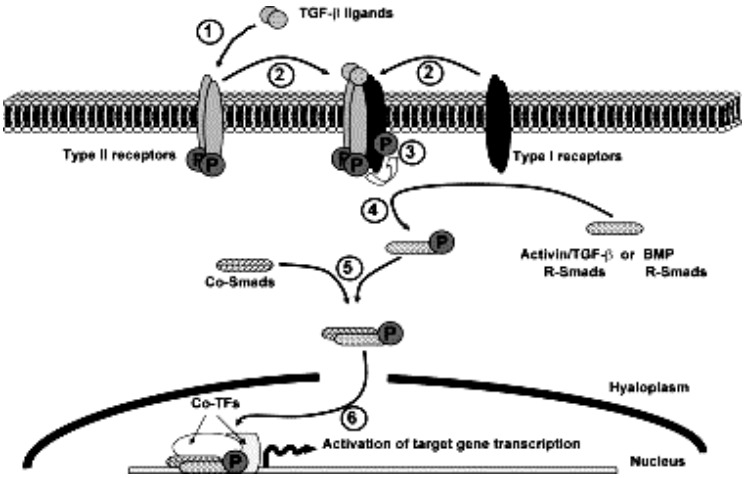

Figure 1-19. Cell signaling pathway of TGF-β superfamily of ligands. (1) Interaction of ligands with constitutively phosphorylated type II receptors. (2) Formation of heterotetramers by recruitment of type I receptors. (3) Transphosphorylation of type I receptor by type II receptor. (4) Recruitment and phosphorylation of active receptor associated Smads. (5) Dimerization with common Smad 4: (6) Transcription of target genes by Smads associated with DNA binding partners (CoTF: Co-transcription factors) (ref. [110])

In addition to Smad signaling, 3 subgroups of MAPK superfamily members, such as ERK1/2, p44/p42 MAPKs, JNK/SAPK and p38 MAPK, are involved in the TGF-β signaling cascade in renal mesangial cells [108]. Another cytokine relevant to TGF-β signaling is CTGF that is induced by TGF-β via consensus Smad and transcription enhancer factor (TEF) elements localized within the CTGF promoter [111]. TGF-β and CTGF synergistically promote cell hypertrophy and ECM deposition, leading to glomerulosclerosis, arteriolar thickening and tubulointerstitial fibrosis [112] and, finally, loss of renal function. In vivo studies by Wang et al. disclosed TGF-β elevation in the glomeruli of STZ-induced diabetic rats by micropuncture techniques [113, 114]. Smad3 has been found to translocate into the glomerular nuclei of diabetic *db/db* mice[115], attesting to the overactivity of the TGF-β system in DN. Using a panselective neutralizing antibody of TGF-β, Ziyadeh et al demonstrated an improvement of diabetic renal hypertrophy, mesangial matrix expansion, and the development of

renal insufficiency[116], although the effect on albuminuria in the diabetic mice is controversial [116, 117]. Therefore, TGF-β signaling is suggested to be central to the ECM pathobiology in DN (figure 1-20) [118].

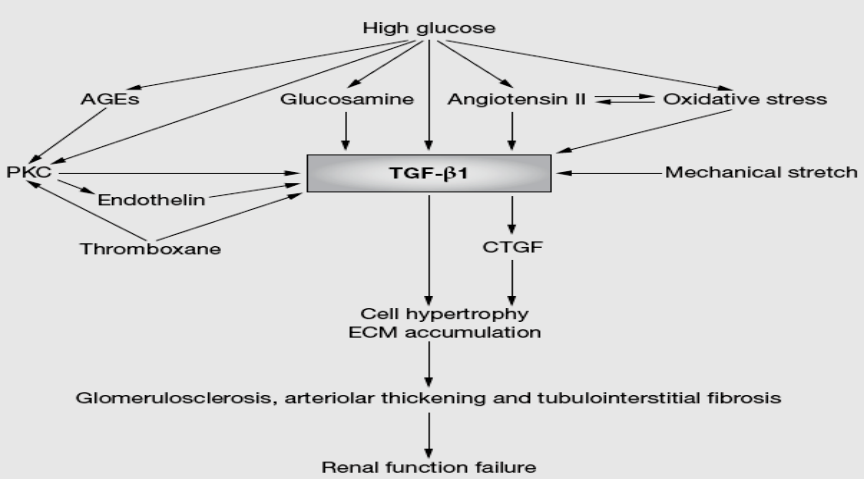

Figure 1-20. Molecular mechanisms of DN (ref. [118])

1.5.1.4 Hypertension and DN

A high prevalence of hypertension is observed in first-degree relatives of DN patients, such that susceptibility to developing DN may be influenced by an inherited predisposition to hypertension [119, 120]. The most effective way to slow the progression of DN appears to be sustained control of hypertension. Moreover, glomerular hypertension is detected by renal micropuncture in experimental diabetes, even if systemic BP is normal, indicating that it may be critical to the initiation and progression of DN [121]. A number of observations support the role of glomerular capillary hypertension in the pathogenesis of DN. For example, the renoprotective effect of angiotensin-converting enzyme (ACE) inhibition in experimental diabetes is partly due to a decrease in glomerular capillary hypertension through vasodilation of the glomerular efferent arterioles [122]. Glomerular capillary hypertension results in changes in glomerular volume, which subsequently stretches structural glomerular components, including mesangial cells and podocytes [123]. Stretching alters the morphology of mesangial cells and decreases podocyte number through diverse mechanisms. One of the consequences is an increase in ECM production, and a

diminution of ECM degradation by the morphologically-changed mesangial cells mediated through TGF-β1 [124, 125]. Mechanical stretching may also stimulate an inflammatory reaction in mesangial cells, which may further contribute to glomerular injury. Furthermore, monocyte chemoattractant protein-1 (MCP-1) and adhesion molecule intercellular adhesion molecule-1 (ICAM-1) have been implicated in DN[126, 127].

Podocytes have also been implicated in DN. These cells are important for the synthesis of GBM matrix proteins, and for the maintenance of glomerular barrier function. At the same time, podocytes are very sensitive to mechanical force[123]. Mechanical stretching can decrease podocyte number through several mechanisms, including reduction of proliferation[128], induction of podocyte apoptosis [129], and podocyte detachment from the GBM. Integrin α3β1 is an adhesion molecule that is primarily involved in podocyte adhesion to the GBM [130]. In conditions of mechanical stress, the expression of integrin α3β1 expression is diminished; consequently, podocyte adhesion to an ECM substrate is reduced [131].

1.5.1.5 Oxidative stress in DN

Under normal circumstances, the high metabolic activity of the kidneys generates a considerable amount of ROS. The urinary environment is remarkably pro-oxidant, and an extensive repertoire of antioxidant enzymes coexists with non-enzymatic free radical scavengers, to balance the local oxidation-reduction environment. Diabetes is accompanied by increased ROS generation in tissues, including the kidneys, involving multiple pathways. Although the origin of increased ROS generation in renal diseases is multifactoral, a major source of renal ROS is a family of non-phagocytic NADPH oxidases that are structurally related to but functionally distinct from NADPH oxidase that is widely studied in neutrophils. The 3 most striking differences are: firstly, non-phagocytic NADPH oxidases seem to generate constitutively low levels of superoxide, even in unstimulated cells; secondly, although enzyme activity can be upregulated in some pathological conditions, measurable

superoxide is still much lower than that found in activated neutrophils; finally, a substantial proportion of the ROS generated in non-phagocytic cells seems to be intracellular, whereas neutrophil superoxide generation during phagocytosis is thought to occur in the extracellular (phagosomal) compartment [132]. Evidence from experimental animal and human studies has suggested that non-phagocytic NADPH oxidases are a major enzymatic source of ROS generation in some pathological conditions [133, 134]. In fact, Zalba et al. postulated that NADPH oxidase was the most important source of superoxide in the vessel wall of experimental hypertension models, including AngII-induced hypertension, renovascular hypertension, and genetic hypertension [134].

NADPH oxidase is a multi-subunit enzyme that catalyzes superoxide production by the 1-electron reduction of O_2 using NADPH as the electron donor: $2O_2 + NADPH \rightarrow 2 \cdot O_2^- + NADP^+ + H^+$. NADPH oxidase was originally found in phagocytic cells. It is an enzyme complex composed of p47phox ("phox" stands for *ph*agocyte *ox*idase), p67phox, p40phox, p22phox, the catalytic subunit gp91phox (also termed "Nox2") [135, 136] and the small GTPase, Rac2. In resting cells, p47phox, p67phox, and p40phox are sequestered to the cytosol, whereas p22phox and gp91phox are located in the membrane, forming a heterodimeric flavoprotein, cytochrome b558. Once the cell is stimulated, p47phox becomes phosphorylated, and the cytosolic subunits form a complex. This complex translocates to the membrane, where it binds with cytochrome b558 to assemble active oxidase, which transfers electrons from NADPH to O_2, forming $\cdot O_2^-$ [137]. Activation of NADPH oxidase also requires the participation of Rac2 (or Rac1) and Rap1A [138] (figure 1-22) [139]. Owing to the recent discovery of gp91phox homologues, members of the Nox (for NADPH-oxidase) family of NADPH oxidases (figure 1-21 [140]) have been designated as Nox1, Nox2 (formerly termed "gp91phox"), Nox3, Nox4, Nox5, Duox1, and Duox2 [141] [142] [143]. They are expressed in many tissues and mediate diverse biological functions.

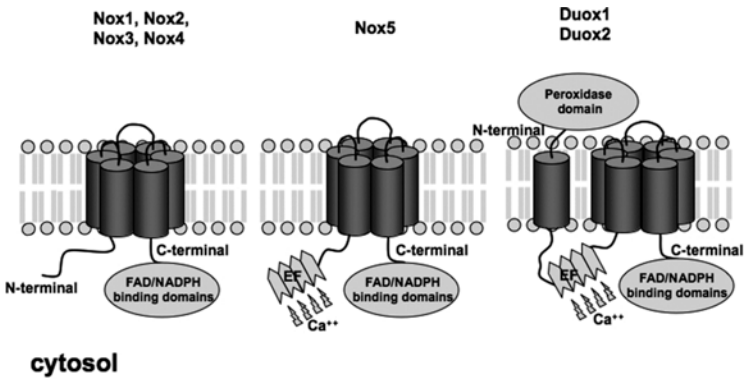

Figure 1-21. Predicted structure of Nox isoforms: All Nox isoforms are predicted to have six transmembrane a-helices containing histidine residues implicated in haem binding. The C-terminal domain binds to FAD and NADPH. Nox5 and Duox contain a calmodulin-like EF domain, while Duoxs additionally have an N-terminal peroxidase homology domain on the extracellular site of the membrane (ref. [140])

Among these homologues, Nox4, originally termed "Renox" (renal oxidase), is abundantly expressed in the kidneys. Recent studies implicated Nox4 in the process of DN. Etoh et al. showed increased expression of Nox4 and $p22^{phox}$ in STZ-induced diabetic rat kidneys. By immunohistochemical analysis they demonstrated that the expression of Nox4 and $p22^{phox}$ was clearly augmented in both distal tubular cells and glomeruli from diabetic rats [144]. They also found that Nox4 and $p22^{phox}$ co-localized with 8-OHdG, a marker of ROS-induced DNA damage. Another experiment by Gorin et al. indicated a causative relationship between Nox4-derived ROS and DN: antisense oligonucleotides were employed to inhibit Nox4 expression, which effectively reduced ROS generation and prevented the development of hypertrophy and increases of fibronectin expression [145].

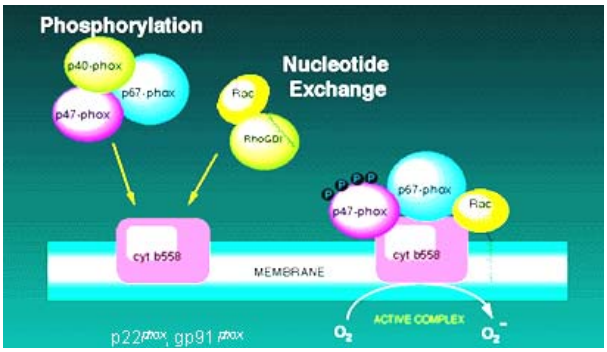

Figure 1-22. NADPH oxidase subunits (ref. [139])

In a review, Ha and Lee postulated that ROS plays a critical role in ECM remodeling in DN. High glucose, AGE, AngII, and TGF-β1 all augment intracellular ROS in renal cells. Additionally, they contribute to heightened ECM synthesis and decreased ECM degradation, leading to glomerular mesangial expansion and tubulointerstitial fibrosis in the diabetic kidney (Table 1-4)[146].

Table 1-4. Evidence for the critical role of ROS in ECM remodeling in diabetic kidney (ref.[146])

ROS are generated by high glucose, AGE, Ang II, and TGF-β1 in renal cells.
High glucose, TGF-β1, and H_2O_2 upregulate ECM synthesis and secretion by renal cells.
High glucose, TGF-β1, and H_2O_2 upregulate PAI-1 expression and downregulate plasmin and matrix metalproteinase activity in renal cells.
AGE, TGF-β1, and H_2O_2 induce EMT (epithelial mesenchymal transition) in tubular epithelial cells.
Antioxidants reverse high glucose and TGF-β1–induced changes in ECM synthesis, PAI-1 and plasmin activity, and TGF-β1–induce EMT.

Another source of intracellular ROS in response to hyperglycaemia is the mitochondria. Kakimoto et al. [147], by detecting of urinary 8-OHdG levels, supposed to be markers of the total systemic oxidative stress in vivo [148], demonstrated that hyperglycaemia increased oxidative mitochondrial DNA damage and mitochondrial DNA deletion in diabetic rat kidneys. Although insulin normalized renal 8-OHdG levels, it did not reverse mitochondrial DNA deletion [147], suggesting that mitochondrial DNA damage may be involved in the pathogenesis of DN. A clinical study investigating the relationship between oxidative DNA damage and tubulointerstitial injury in T2D patients with DN, indicated that urinary 8-OHdG levels were higher in T2D patients than in their controls. In addition, 8-OHdG levels tended to rise with the severity of glomerular lesions, and increased with the severity of tubulointerstitial lesions in T2D patients[149]. Furthermore, Houstis et al. recently demonstrated a causal role of ROS in both in vitro and in vivo models of insulin resistance[150].

1.5.1.6 Peroxisome proliferator-activated receptors (PPARs) and DN

PPARs belong to the steroid/nuclear receptor superfamily. Three PPAR isoforms have been identified and characterized: PPARα, PPARβ/δ and

PPARγ[151] [152, 153]. PPARs are critical in regulating diverse biological processes, such as lipid metabolism, adipogenesis, insulin sensitivity, immune responses, and cell growth and differentiation[152-155]. They have also been shown to participate in the pathogenesis of human diseases, including insulin resistance, glucose intolerance, obesity, dyslipidemia, hypertension, atherosclerosis, and microalbuminuria [156-158]. Although the 3 PPAR subtypes have overlapping biology, each isoform has unique patterns of tissue distribution, ligand selectivity and biological effects. Figure 1-23 shows the general structure of PPAR and 3 isoforms[159].

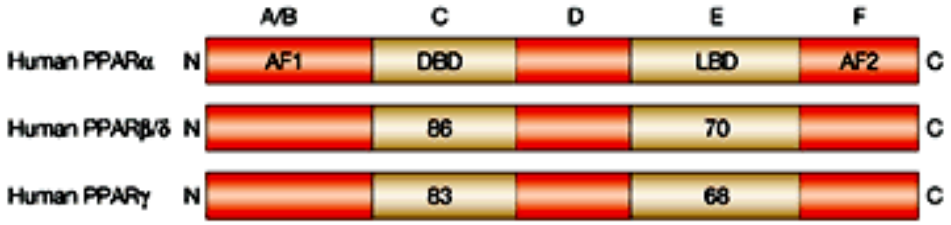

Figure 1-23. PPAR structure. Similar to other nuclear hormone receptors, the peroxisome proliferator-activated receptors (PPARs) contain 5 distinct regions. The 2 most conserved regions are C and E, which consist of a highly conserved DNA-binding domain (DBD) and a moderately-conserved ligand-binding domain (LBD), respectively. The amino-terminal A/B domain contains the AF1 ligand-independent activation domain. An AF2 ligand-dependent activation domain is located in the F region, whereas the D domain consists of a variable hinge region. The numbers shown in the LBD and DBD are the percentage amino-acid identity of human PPARβ/δ and PPARγ1 with respect to human PPARα.(ref.[159])

(a) PPARα and DN

In the kidneys, PPARα is highly expressed in the PTs and medullary thick ascending limbs [160, 161] playing a critical role in renal energy homeostasis [162]. PPARα gene expression has been documented to increase markedly in the diabetic kidney [163]. Though the underlying mechanisms by which PPARα agonists attenuate DN remain uncertain, several recent clinical studies have provided clear evidence that the fibrate class of PPARα agonists confers a renoprotective effect in patients with T2D [164, 165]. The protective influence of PPARα agonists can be multi-functional; for instance, PPARα may be systemically implicated in attenuating insulin resistance, glycaemic control and lipid toxicity in β-cell islets [166-168]. In the kidneys, PPARα activation may have direct impact on mesangial cells. Its activation was found to block oxidant stress-

induced TGF-β1 expression, attenuating glomerulosclerosis [169]. Kamijo et al. [170] reported that starved PPARα-null mice showed increased urine albumin excretion and albumin accumulation in PTs, suggesting that PPARα can promote albumin reabsorption and degradation in PTs,. Taken together, the evidence supports the view that PPARα is important for insulin-sensitivity regulation, and could represent a promising target in the treatment and prevention of T2D.

(b) PPARγ and DN

PPARγ is mainly expressed in the distal medullary collecting ducts and expressed less so in glomeruli and the renal microvasculature [160, 171]. It can also be detected in proximal tubules [154]. A group of PPARγ agonists known as thiazolidinediones (TZD), which includes troglitazone, rosiglitazone, and pioglitazone, has beneficial effects on urinary albumin excretion and/or renal fibrosis in animal models of insulin resistance, T2D, and hypertension [172]. Many clinical studies have also demonstrated that PPARγ agonists protect renal function by reducing albuminuria in T2D patients with or without hypertension [172-175]. Furthermore, clinical investigations have revealed that troglitazone, rosiglitazone and pioglitazone produce superior renoprotection and similar glycaemic control in T2D patients compared with insulin and other oral hypoglycemic agents, including metformin, glyburide and glibenclimide[176, 177] [178, 179]. TZDs even show better renal protection than ACE inhibitors against renal injury in T2D rats[180]. In vitro data also support the benefits of PPARγ agonists in mesangial cells [181, 182], glomerular podocytes [183],and PTCs [184, 185]. Although T1D has mechanism(s) different from T2D, studies have established that TZD PPARγ agonists are as effective in attenuating DN in T1D as they are in T2D [186, 187].

In summary, these results indicate that PPARγ activation exerts anti-proliferative, anti-fibrotic and anti-inflammatory actions in many renal cells both in humans and animals, thereby ameliorating the progression of glomerular and tubulointerstitial fibrosis in hyperglycaemic states. With these renal protective features, PPARγ is a promising target for treating glomerular fibrotic diseases,

especially DN, but its side effects such as weight gain and fluid retention [188] need to be overcome to guarantee its long-term beneficial outcomes.

(c)PPAR β/δ and DN

Unlike PPARα, and PPARγ, little is known about the physiological roles of PPAR β/δ, probably because of a wide tissue distribution pattern and lack of a PPAR β/δ ligand in current clinical use[189]. Renal PPARβ/δ is expressed diffusely in the cortex and medulla, including mesangial cells, medullary interstitial and stromal cells [190] [160].

Recently, Proctor et al. examined PPARβ/δ expression in 2 T1D mouse models, Akita and OVE26. In both models, they found that renal PPARβ/δ expression was greatly suppressed, which might have contributed to renal lipotoxicity because of reduced fatty acid oxidation [191]. An in vitro study by Hao et al. demonstrated that PPARβ/δ overexpression provided protection against hypertonicity-induced cell death in cultured medullary interstitial cells, implying that PPARβ/δ was a critical survival factor in the kidneys [192]. Therefore, reduced renal PPARβ/δ expression is possibly one of the pathways involved in diabetic kidney injury, and PPARβ/δ agonists may confer renal protection against DN and other kidney diseases.

1.5.2 Apoptosis and diabetic kidneys

1.5.2.1 General apoptosis pathways

Apoptosis was first reported in 1972 by Kerr et al. [193] who described a morphologically distinct form of cell death, in which a controlled sequence of events (or programs) led to the elimination of cells without releasing harmful substances into the surrounding area. In mammalian cells, the apoptotic response is mediated through either the intrinsic or the extrinsic pathway, depending on the origin of the death signal. The extrinsic pathway begins outside a cell, where conditions in the extracellular environment determine whether it must undergo apoptosis. This pathway is also called the death receptor pathway, since apoptosis is induced through the binding of death ligands to cell surface death receptors. Adapter-molecules like FADD (Fas-

associated via death domain), TRADD (tumor necrosis factor receptor-1-associated death domain) or Daxx (a death-domain-associated protein, which binds specifically to the Fas death domain) contain death domains so that they can interact with death receptors and transmit the apoptotic signal to the death machinery. The best-characterized death receptors are Fas and TNFR1 (tumor necrosis factor receptor-1) [194]. FasL (Fas ligand) binds to Fas and causes oligomerization of its receptor, Fas. This results in clustering of the death domains and binding of co-factor FADD. FADD protein binds, via its DED (death effector domain) motif, to a homologous motif in pro-caspase-8. The complex of Fas, FADD and pro-caspase-8 is called DISC (death-inducing signaling complex). Upon recruitment by FADD, pro-caspase-8 is activated through self-cleavage. Active caspase-8 then activates downstream caspases (caspase-3 and -7), committing the cell to apoptosis [195].

In addition to receptor-mediated apoptosis, another pathway activated by various forms of cellular stress is the intrinsic pathway. In this pathway, since the mitochondrion is the central link in the chain of reactions, the intrinsic pathway is also called the mitochondrial pathway. Stress-induced apoptosis occurs by a mechanism that involves altering mitochondrial permeability, and, as a result, cytochrome c, is released from the mitochondria, binds to Apaf-1 to form the apoptosome together with pro-caspase-9. Pro-caspase-9 cleavage elicits the formation of active caspase-9, and this is followed by the activation of pro-caspase-3 to caspase-3. Caspase-3 activation evokes the physical manifestation of apoptosis, such as DNA degradation, chromatin condensation, etc.

Other proteins released from damaged mitochondria, such as SMAC (second mitochondria-derived activator of caspase)/DIABLO (direct inhibitor of apoptosis (IAP)-binding protein with low pI (isoelectric point)), AIF (apoptosis-inducing factor), and OMI/HTRA2 (high temperature requirement protein-A2), counteract the effect of IAPs, which normally bind and prevent pro-caspase-3 activation. Recent studies have demonstrated that the nuclease Endo-G (endonuclease-G) is specifically activated by apoptotic stimuli and is able to

induce nucleosomal fragmentation of DNA independently of caspase and DFF (DNA-fragmentation factor)/CAD (caspase-activated DNAse). Endo-G is a mitochondria-specific nuclease that translocates to the nucleus and cleaves chromatin DNA during apoptosis. AIF is also attributed a role in apoptosis, becoming active upon translocation from the mitochondria to nuclei, where it initiates chromatin condensation and large-scale DNA fragmentation [196].

There is evidence that the extrinsic and intrinsic pathways are linked, but this has not been proven definitively. One hypothesis is the caspase-8-mediated cleavage of BID (a BH3-only member of the Bcl-2 family) to truncated BID (tBID). While full-length BID is localized in the cytosol, tBID translocates to the mitochondria and transduces apoptotic signals from the cytoplasmic membrane to the mitochondria, causing cytochrome c release, loss of mitochondrial membrane potential, cell shrinkage, and nuclear condensation in a caspase-dependent fashion [197]. Figure 1-24 describes the apoptotic pathways [198].

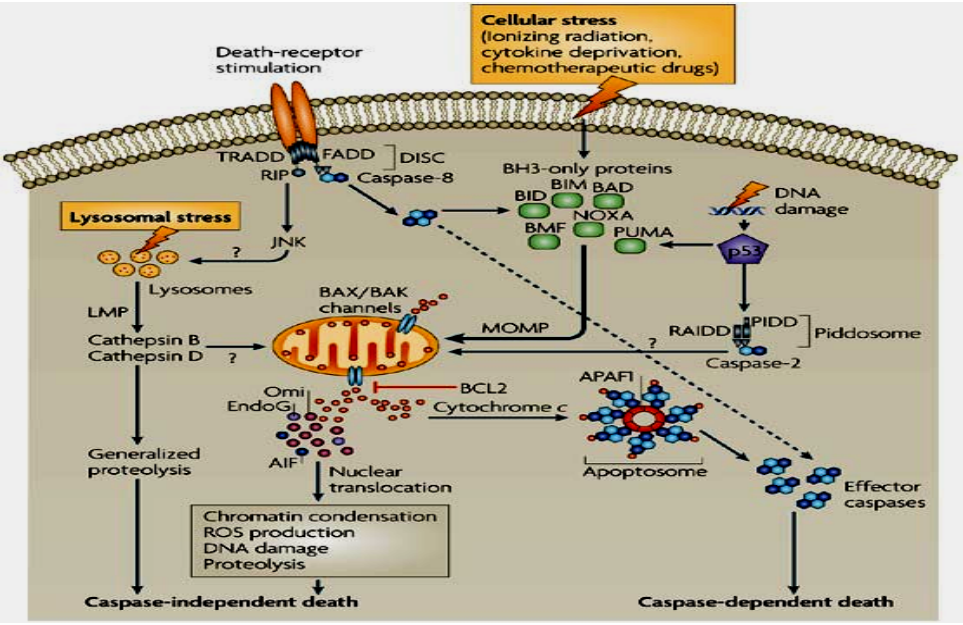

Figure 1-24 Intrinsic and extrinsic apoptosis pathways (ref. [198])

1.5.2.2 ER stress and apoptosis

The ER is an organelle where newly-synthesized secretory or membrane proteins form their proper tertiary structure by post-translational modification,

folding, and oligomerization; only correctly-folded proteins can be transported to the Golgi apparatus. The accumulation of misfolded proteins in the lumen of the ER is a condition called ER stress, which is a risk for living cells. To deal with the stress, a self-protective mechanism in mammalian cells, called the unfolded protein response (UPR) is triggered. The UPR causes the following: (1) attenuation of protein synthesis to prevent further accumulation of unfolded proteins; (2) transcriptional induction of ER chaperone genes to enhance folding capacity; and (3) transcriptional induction of ER-associated degradation (ERAD) component genes to increase ERAD ability [199]. If the stress cannot be resolved by these mechanisms, and the cells are exposed to prolonged or strong ER stress, induction of apoptosis ensues to ensure survival of the organism. UPR is mediated through 3 ER trans-membrane receptors: pancreatic ER kinase (PKR)-like ER kinase (PERK) [200-202], activating transcription factor 6 (ATF6) [203-205] and inositol-requiring enzyme-1 (IRE1) [206-208]. The 3 ER stress receptors are in an inactive state through their association with the ER chaperone GRP78. ER stress causes the dissociation of GRP78 from the receptors, leading to their activation and triggers the UPR. In addition, accumulating evidence suggests that these UPR mediators are also fundamental to ER stress-induced apoptosis (Figure 1-25) [199].

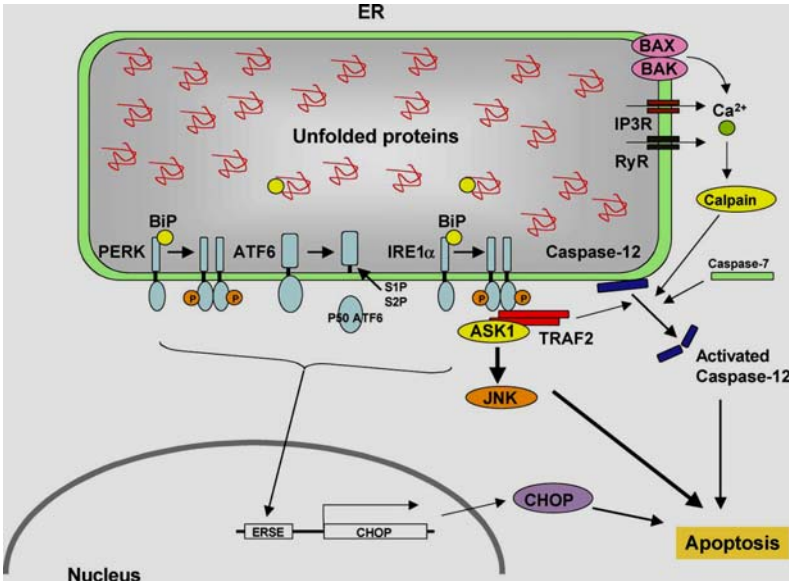

Figure 1-25. Mechanisms of apoptosis in response to ER stress (ref. [199])

In humans, ER stress contributes to ß-cell loss in different diabetes subtypes, such as Wolcott-Rallison syndrome, a rare disorder characterized by neonatal or early-infancy, insulin-dependent diabetes [209]. More direct evidence for a role of ER stress in type 2 diabetes (T2D) comes from a recent study by Laybutt *et al.* [210] showing that higher staining intensity was observed for glucose-regulated protein 78/binding immunoglobulin protein (BiP) (GRP78/BiP), CHOP ((C/EBP (CCAAT/enhancer-binding protein) homologous protein) from β-cells in pancreatic sections from T2D patients compared to non-diabetic pancreatic tissue. The CHOP pathway is characterized by apoptosis in ER stress. CHOP/GADD153 (growth arrest and DNA damage-153), a transcription factor, is barely detected under physiological conditions, but is strongly induced in response to ER stress [211]. Upon ER stress, ATF6 and PERK are activated and CHOP/GADD153 expression is evoked by the ATF6 and PERK pathways [205, 212, 213]. PERK transmits signals by eIF-2a phosphorylation, which can elicit ATF4 translational upregulation [200]. ATF6 is cleaved by ER stress-induced proteolysis and releases an N-terminal cytosolic domain (p50ATF6). X-box-binding protein 1 (XBP-1), ATF4 and p50ATF6 work as a transcription factor to increase CHOP mRNA [205]. Zinszner et al. demonstrated that ER stress induced less apoptosis in CHOP-/- cells, suggesting that the CHOP pathway is a major regulator of ER stress-induced apoptosis [214]. Studies examining the target genes of CHOP have identified that it activates the transcription of GADD34, ER oxidoreductin-1 (ERO1), DR5 (death receptor 5), and carbonic anhydrase VI, which seem to be relevant to apoptosis. GADD34 can interact with protein phosphatase-1 (PP1) and causes it to dephosphorylate eIF2α and thus release the translational block promoting ER protein biosynthesis [215, 216]. GADD34 expression correlates with apoptosis induced by various signals, and its overexpression can initiate or enhance apoptosis [217]. ERO1, which encodes an ER oxidase, on the other hand, hyper-oxidizes the ER. Observations by Marciniak et al. indicate that ER oxidation might also favor protein malfolding [218]. DR5, encoding a cell surface death receptor, which may activate caspase cascades to induce apoptosis, is

up-regulated by ER stress in cancer cells. Furthermore, DR5 was discovered to be directly regulated by CHOP at the transcriptional level, and CHOP is responsible for the induction of DR5 by ER stress. Moreover, DR5 was found to be a critical regulator of ER stress-induced apoptosis of human carcinoma cells [219]. A heterozygous Akita mouse, which is a widely adopted type I diabetes model, would delay diabetes development by 8 to 10 weeks in homozygous disruption of CHOP mice [220]. To summarize, these data have improved our understanding of how CHOP promotes apoptosis.

Caspases are well-known pro-apoptotic components. Caspase-12 has been identified as a molecule which selectively initiates apoptosis in response to ER stress [221]. Caspase-12 is localized on the ER membrane; once specifically activated by ER stress, it translocates to the cytoplasm. Then, it activates caspase-9, which, in turn, activates caspase-3 [222], leading to cell death. Nakagawa et al. reported that caspase-12$^{-/-}$ mice are resistant to ER stress-induced apoptosis but sensitive to other death stimuli, suggesting that caspase-12 is a regulator specific to ER stress-induced apoptosis. They proposed that pro-caspase-12 is cleaved by m-calpain in response to calcium release from the ER [223]. Yoneda et al. established that activated IRE1α recruits TNFR-associated factor-2 (TRAF2), which interacts with pro-caspase-12 and promotes its clustering and activation [224]. Rao et al [225] found that caspase-7 was involved in caspase-12 activation. However, the involvement of caspase-12 in apoptosis of human cells needs to be elucidated, as the human caspase-12 gene contains several mutations critical to its function [226]. Therefore, identification of a functional homologue of caspase-12 in humans and its function in ER stress-induced apoptosis is an issue to be addressed.

Bcl-2 family proteins can be divided into 2 groups: anti-apoptotic and pro-apoptotic. The anti-apoptotic group includes Bcl-2 and Bcl-XL, while the pro-apoptotic group is sub-divided into a BH3-only class (including Bid and Bim) and a multidomain class (including Bax and Bak). Generally, pro-apoptotic members of the Bcl2 family seem to be recruited to the ER surface to activate caspase-12, whereas the anti-apoptotic members inhibit this recruitment, although the exact

relationship between these factors is still unclear. McCullough et al. discovered that Bcl-2 is down-regulated by CHOP upon ER stress, leading to augmented oxidant injury and apoptosis [227]. Another group, Contreras et al. reported that overexpression of Bcl-2 inhibited the activation of caspase-12 and apoptosis during ER stress [228]. Bax and Bak are normally located in the cytosol and translocate to the mitochondria in response to various apoptotic stimuli Bax and Bak, recently found in the ER membrane as well as the mitochondrial membrane, are essential for ER stress-induced apoptosis by maintaining the homeostasis of ER Ca^{2+} [229] [230]. Wei et al. observed that cells deficient in both Bax and Bak are resistant to multiple apoptotic stimuli, including ER stress stimuli, such as thapsigargin and tunicamycin [231]. Testing diverse ER stress inducers, Zong et al. noted increases in the active forms of Bax and Bak, indicating that Bax and Bak underwent conformational changes from their inactive to active forms in response to ER stress stimuli. Bax and Bak on the ER can directly affect ER Ca^{2+} stores by depletion of ER Ca^{2+}. They also found that ER stress treatment did not induce caspase 12 activation in *bax-/- bak -/-* cells, suggesting that caspase 12 activation is Bax / Bak dependent. Although no relationship between ER Ca^{2+} depletion and caspase 12 cleavage was investigated and no other detailed pathway through which caspase 12 could be activated was further demonstrated. Moreover, CHOP was induced by ER stress treatment in $bax^{-/-}$ $bak^{-/-}$ cells, indicating that cells deficient in Bax and Bak are still responding to ER stress-induced UPR [230]. In contrast to most BH3-only proteins, which target mitochondria, BIK (Bcl2-interacting killer) is integrated almost exclusively in the ER membrane [232, 233]. By using human H1299 cells or H1299 cells stably overexpressing Bcl-2 infected with an adenovirus containing BIK (Ad BIK), Matha et al. found that Ad BIK was able to trigger Bak ER recruitment and oligomerization [234].

Finally, another important pathway in apoptosis is the JNK pathway. Urano et al. showed that activated IRE1 on the ER membrane recruits TRAF2, thereby activating c-Jun amino-terminal kinase (JNK) [235]. In addition, IRE1 overexpression induced apoptosis in HeLa cells [206]. Furthermore, IRE1

activation and complex formation of endogenous TRAF2 and apoptosis signal-regulating kinase 1 (ASK1) were observed in thapsigargin-treated primary neurons by Nishitoh et al. This group studied 293 cells transfected with Myc-ASK1, HA-IRE1, and Flag-TRAF2. They discerned that ASK1 was associated with IRE1 only in the presence of TRAF2 plus thapsigargin, which suggested that ER stress induced the formation of an IRE1-TRAF2-ASK1 complex on the ER outer membrane and thus activated the ASK1-JNK pathway [236], which subsequently resulted in apoptosis.

Apoptosis is a common reason for pathological changes in the diabetic kidney. All the pathways that have been discussed above appear to be involved in DN. They will be elaborated further in the following paragraphs.

1.5.2.3 Apoptosis in diabetic glomeruli

In a glomerulus, different cell types play different functional roles. Excessive apoptosis of any cell type can result in abnormal renal function in the diabetic condition. Among these cells, the apoptosis of podocytes and mesangial cells is of great interest.

Podoctyes are an important member of the glomerular filtration barrier, and it is intriguing that in DM their numbers and density are reduced; in some cases, they also exhibit effacement of foot processes. Declining podocyte numbers could be the result of their detachment from the GBM into the urinary space or their apoptosis. The scenario that podocytes detach from the GBM does not exclude a role for apoptosis, since cells may first detach and then undergo apoptosis because of interruption of viability cues deriving from cell-matrix interactions [237]. Emerging evidence from animal experiments is supporting the notion that DM results in higher rates of podocyte apoptosis. For example, Susztak et al. reported that podocyte apoptosis increased with the onset of hyperglycaemia in Akita mice (an animal model of T1D) and db/db mice (T2D). In addition, they discovered that podocyte apoptosis coincided with the onset of urinary albumin excretion but preceded significant podocyte loss from the glomerulus. Their *in vitro* study further demonstrated a role for

hyperglycaemia-induced increased ROS in podocyte apoptosis and podocyte depletion, and suggested that podocyte apoptosis/depletion represented a novel early mechanism leading to DN [238]. Menini et al. [236] also showed that glomerular cell apoptosis was significantly augmented in STZ-induced diabetic rats at 4 months and to an even greater extent at 6 months, with podocytes accounting for 70% of apoptotic cells. Even after 2-week induction of diabetes by STZ, a significant reduction in podocyte/glomerular cross-section density was found by Siu et al. [239]. They also observed a similar decline in podocyte number in STZ diabetes in C57BL/6 mice at 2 weeks [239].

Unlike podocytes, the effect of hyperglycaemia on mesangial cells seems to appear later, causing mesangial injury. In fact, by evoking pathways leading to apoptotic cell death, hyperglycaemia could be one of causes for the loss of cellular components in glomerulosclerosis. Increased mesangial cell apoptosis also correlates with ECM accumulation in hyperglycaemia-induced segmentation of the glomerular tuft [240]. Several groups have studied mesangial apoptosis induced by hyperglycaemia. Kang et al reported that high glucose stimulated caspase-3 cleavage and DNA fragmentation in cultured human mesangial cells, and ROS was implicated in the activation of apoptosis [241]. Yamagishi et al. demonstrated that apoptosis of cultured human mesangial cells was induced by AGEs [242]. In addition, TGF-β1, which is activated in diabetes, was shown to induce apoptosis in cultured mesangial cells [243]. Mishra et al. further established that chronic hyperglycaemia caused apoptosis in human mesangial cells and activated a caspase-9-dependent intrinsic pathway of pro-apoptotic signaling. Moreover, they also detected apoptotic mesangial cells in db/db mice [244]. Very recently, Jung et al. observed significantly increased apoptotic cells in diabetic glomeruli and in high-glucose-stimulated mesangial cells compared to control glomeruli and mesangial cells cultured in normal glucose medium, respectively [245].

1.5.2.4 Apoptosis in diabetic tubules

Diabetic kidney disease was originally described as glomerulopathy

associated with diffuse or nodular glomerulosclerosis. However, less than one-third of diabetic patients with microalbuminuria have the pathological alterations mentioned above [73]. On the other hand, functional and structural changes in PTs correlate better with the progression of DN. In fact, numerous experiments have revealed that apoptosis in PTs is a pivotal change that results in the deletion of individual renal tubular cells and tubular atrophy. More than 10 years ago, Ishii et al. infused 10% glucose, 10% mannitol or 0.9% saline solution into male Wistar rats for 300 minutes via the left cervical vein, which led to PT apoptosis [246], implying that hyperglycaemia caused tubular apoptosis. Apoptosis has also been detected in renal cortical tubular cells from diabetic db/db mice [247]. As well, Kumar et al observed apoptosis in STZ-induced diabetic rats in tubular and interstitial cells by terminal deoxynucleotidyl Transferase dUTP nick end labeling (TUNEL) assay, DNA laddering and electron microscopy [248]. In vitro studies further support the in vivo findings. For instance, by employing annexin V staining and TUNEL assay, Verzola et al. showed that exposure of HK-2 cells to 30 mM glucose for 48 hours caused a significant increase of apoptosis compared to 5.5 mM glucose [249]. In another PTC line, LLC-PK1 cells, Allen et al. reported that LLC-PK1 cells exposed to high D-glucose (25 mM) for up to 48 hours manifested increased DNA fragmentation, caspase-3 activity, and annexin-V staining as well as decreased expression of X-chromosome-linked inhibitor of apoptosis protein (XIAP) compared to the controls (5 mM D-glucose) [250].

Likewise, our laboratory demonstrated that high glucose also induced apoptosis in renal PTs in vitro and in vivo. In rat immortalized renal proximal tubular cells (IRPTCs), we observed that high glucose evoked ROS generation and IRPTC apoptosis. Furthermore, we noted apoptotic renal proximal tubular cells (RPTCs) in both STZ-induced diabetic mice and db/db mice [251] [252] [253]. Notably, tubular apoptosis can be prevented by antioxidants [249], suggesting a cause-and-effect relationship between ROS generation and early apoptotic changes. Moreover, proteinuria, developed during the progression of diabetes, can elicit tubular apoptosis by stimulating the mitochondrial apoptotic

pathway and/or evoking ER stress, and subsequently activating caspase-12 [254][255].

1.6 The renin-angiotensin system (RAS)

Since the first identification of renin by Tigerstedt and Bergmann in 1898, the RAS has been studied extensively. The RAS is an extracellular hormonal system that plays an important role in regulating blood volume and systemic vascular resistance. Angiotensinogen (Agt), the sole substrate of the system, is released from the liver and cleaved in the circulation by renin secreted from the JG apparatus of the kidneys, forming the decapeptide AngI, which is then activated to the octapeptide AngII by ACE, a membrane-bound metalloproteinase. ACE is predominantly expressed at high concentrations on the surface of endothelial cells in the pulmonary circulation. AngII, the main bioactive peptide of the RAS, acts on 2 receptors, AngII subtype I receptor (AT1R) and AngII subtype 2 receptor (AT2R), which are expressed in cardiovascular tissues as well as in other tissue types. AT1R confers most of the classical actions of AngII, such as vasoconstriction, stimulation of aldosterone release from the adrenal glands and vasopressin from the posterior pituitary to increase sodium and fluid retention by the kidneys, as well as stimulation of the sympathetic nervous system via receptors in the brain. In rodents, AT1R has 2 isoforms: AT1RA and AT1RB; AT1RA mediates most of these actions.

Recent findings have significantly expanded knowledge of the RAS. A homologue of ACE, ACE2, was discovered and shown to cleave AngII, yielding Ang1-7. Ang1-7 has multiple actions that mostly counteract those described for AngII, as reported by Donoghue et al. and Tipnis et al. separately in 2000 [256] [257]. ACE2 shares 44% sequence homology with ACE, but conventional ACE inhibitors do not block ACE2 activity. Furthermore, AngI can be effectively cleaved to form Ang1-7 by neutral endopeptidase (neprilysin; NEP) [258]. This is an important consideration as ACE inhibition results in higher levels of AngI. Increased levels of Ang1-7 may contribute to antihypertensive effects [259]. Santos et al. discovered that Mas proto-oncogene is a receptor for Ang1-7 and

that the ACE2-Ang1-7-Mas axis counter-regulates the actions of the classical RAS [260] [261].

Acute stimulation or depression of the RAS is mediated primarily by the regulation of renin release and expression. Agt expression had not been regarded previously as an important determinant of AngII formation [260]. However, recent evidence demonstrates that changes in Agt concentration can indeed affect the rate of AngII formation [262] [263].

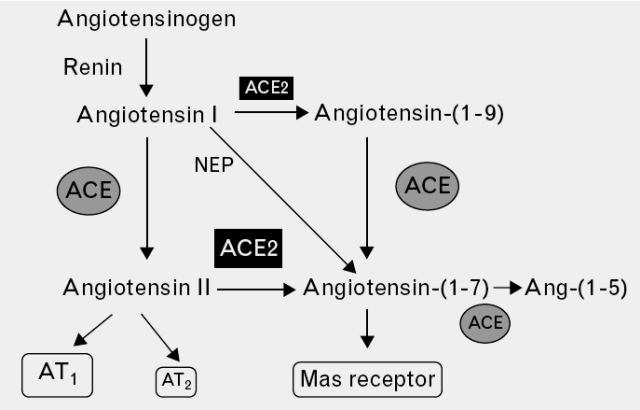

Figure 1-26 Renin–angiotensin system(ref. [264])

1.6.1 Local RASs

The existence of local RASs, independent of the circulating RAS, became apparent in the early 1970s. Studies have shown that AngII is not only generated in the circulation by renin and ACE, but is also produced locally in numerous organs, including the kidneys, vessels, heart, adrenal glands, eyes, testes, brain, etc. In contrast to the circulating RAS, which acts as an endocrine system, forming AngII extracellularly and delivering it by blood to various target tissues. AngII generated by local RASs may have both paracrine and autocrine activities [265]. It induces inflammation, cell growth, mitogenesis, apoptosis, migration, differentiation and regulates the gene expression of bioactive substances as well as activates multiple intracellular signaling pathways. Importantly, the effects of local Ang II may contribute to tissue injury [266].

The existence of the intrarenal RAS (Figure 1-27) [267] has been investigated extensively. RPTCs are reported to express both mRNA and

protein for all components of the RAS [268] [269] [270]. Animal experiments have provided convincing evidence that in the lumen of PTs, AngII levels are in the nanomolar range, which is about 1,000-fold greater than in plasma (picomolar range). In addition, components of the RAS have also been detected in glomeruli in both humans and animals. AngII concentrations in glomerular ultrafiltrate are significantly higher than in plasma [271] [272] [273] [274]. It has been demonstrated that AngII produced within the kidneys has an important regulatory effect on renal hemodynamics and functions [275]. Further studies by Navar et al. showed that reduced renal function and structural changes were associated with over-production of intrarenal AngII, leading to the development of hypertension and renal injury [276] [277].

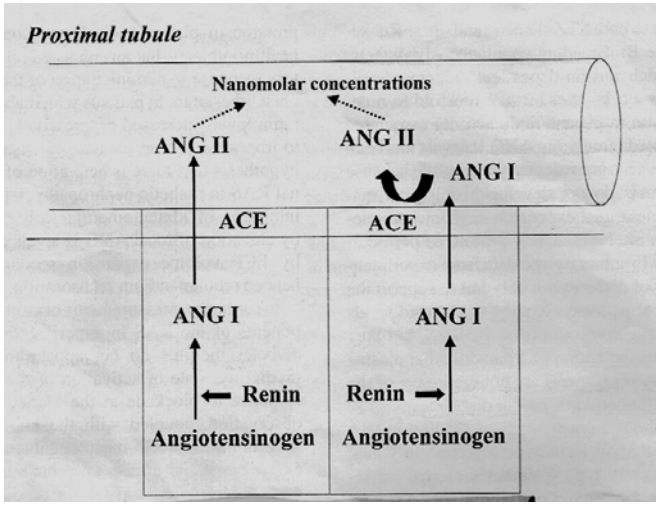

Figure 1-27 Model for the production of AngII by proximal tubular cells (ref,[267])

1.6.2 Intrarenal RAS activation and hypertension development

1.6.2.1 Animal models of RAS activation and hypertension

The RAS regulates BP. Recently, the effects of local tissue RAS on BP regulation and organ damage have drawn much attention. To better understand the role of the local RAS in the pathogenesis of hypertension, Tg animal models that either overexpress or have KOs of specific components of the RAS have been generated.

1.6.2.1.1 RAS KO mice

(a) Mice lacking Agt

More than 10 years ago, Tanimoto et.al.[278] created Agt-deficient mice and found that they had significantly lower BP than wild-type mice (Agt-deficient mice: 66.9 ± 4.1 mmHg *vs.* wild-type mice: 100.4 ± 4.4 mmHg, C57BL/6 strain). Later, Ishida et al. [279] reported that out of 21 newborn Agt KO mice, only 4 survived until weaning, suggesting an important role for Agt in development. In addition, using Agt-deficient mice, Niimura et al. [279], showed that Agt is required in the development and maintenance of kidney morphology. Altogether, these findings clearly indicate that Agt is an essential factor for kidney maintenance and development.

(b) Mice lacking renin

Like humans, C57BL/6 mice have 1 renin gene, *Ren1c*, which makes them a suitable model for research involving changes in renin expression [280]. Takahashi et al. [281] established that mice completely lacking *Ren1c* presented hydronephrosis, polyuria and were unable to concentrate urine. Kidney histological changes in *Ren1c*$^{-/-}$ mice were similar to those seen in Agt KO mice. The BP of *Ren1c*$^{-/-}$ mice was lower than that of their wild-type counterparts at age 4 months. They also manifested metabolic acidosis, high plasma creatinine and blood urea nitrogen, indicating a substantial reduction of the GFR. As well, they demonstrated decreased survival.

(c) Mice lacking ACE1

ACE1-deficient mice display markedly reduced SBP and pathological changes in the kidneys. The renal papilla is dramatically curtailed, and the intrarenal arteries exhibit vascular hyperplasia associated with perivascular inflammatory infiltrates. The renal function of these ACE1-null mice is also affected, exhibiting an inability to concentrate urine and showing an abnormally low urinary sodium to potassium ratio [282]. In agreement with these findings, studies from other groups have revealed that in the complete absence of Agt, ACE1 causes a significant decrease of BP [283] [284].

(d) Mice lacking ACE2

In addition to classical ACE, 3 mammalian homologues of ACE, namely

ACE2, collectrin (collecting duct-specific transmembrane glycoprotein, a novel homolog of ACE2 [285]) and ACE3 have been described recently [257] [286] [287]. Among these 3 homologues of ACE, ACE2 is the only known homologue with enzymatic activity; it is abundantly expressed in the kidneys and may counterbalance ACE activity by promoting Ang II degradation to the vasodilator peptide Ang1-7 [288] [289] [290]. To assess the role of ACE2 in kidney disease, Oudit et al. [291] deleted ACE2 in mice (*ACE2-/-*), and investigated the impact of ACE2 deletion on kidney structure and function. Their data disclosed that loss of *ACE2* was associated with the development of gender-specific and age-dependent glomerulosclerosis in male *ACE2* null mice that was mediated by AngII. Gurley and co-workers observed a modest increase of BP (7 mmHg increase) in ACE2-deficient mice with a C57BL/6 background. After acute AngII infusion, plasma AngII concentrations increased almost 3-fold in ACE2-deficient mice compared to the controls [292]. Their study suggested that ACE2 is a functional component of the RAS, metabolizing AngII and thereby contributing to BP regulation.

(e) Mice lacking AT1RA and/or AT1RB

In contrast to humans, 2 subtypes of AT1R, AT1RA and AT1RB, have been identified in rodents [293] [294]. In gene-targeting experiments, it has been shown that the AT1RA is predominantly involved in the regulation of vascular tone in the periphery as well as in the pressor response of the central nervous system (CNS). AT1RB, on the other hand, contributes to the dipsogenic response to AngII in the CNS [295] [296] [297]. Indeed, Oliverio et al. [298] demonstrated that mice lacking AT1RB were indistinguishable from wild-type littermates by simple observation. No pathological changes were found in their major organ systems, including the heart and the kidneys. This finding is consistent with that of Chen and colleagues [296]. In mice lacking both AT1RA and AT1RB, however, Oliverio and colleagues observed a significant reduction of BP, impaired body and kidney growth, fertility as well as abnormalities of kidney structure, such as atrophy of the inner medulla, severe thickening of the arterial walls within the kidneys, focal areas of cortical interstitial inflammation,

fibrosis, and tubular atrophy [298].

(f) Mice lacking AT2R

AT2R have also been targeted in mice. In contrast to BP reduction in AT1RA KO mice, Ichiki et al. [299] noted a modest elevation of BP in mice lacking AT2R, further confirming that most of the vasoconstrictive effects of AngII are mediated by AT1RA. Gross et al. [300] also reported that by tail-cuff blood pressure measurement, AT2 knockout mice had a 17mmHg increase of BP. However, AT2R-deficient mice appeared to be normal, and no renal developmental abnomalies were described. Moreover, the GFR was normal in these animals [301].

(g) Substitutes of homozygous RAS KO mice

The strong or even lethal effects of the complete absence of individual components of the RAS preclude the use of homozygous KO mice. In contrast, heterozygous KOs of RAS provide better tools for the study of RAS effects on BP. For instance, mice heterozygous for null mutations in Agt were generated by Smithies and colleagues [302], and Sugaya et al. created another model of mutant mice with targeted replacement of AT1RA loci. Chronic hypotension was observed in these heterozygous mutant mice with 10 mmHg lower SBP than in their wild-type littermates [303], although the reduction of BP was less than that of homozygous mice.

1.6.2.1.2 Tg mice overexpressing systemic or intrarenal RAS

(a) Tg mice overexpressing systemic RAS

Another common way to study the RAS is to investigate animals with overexpressed genes of RAS components. Hatae et al. demonstrated that the RAS reaction is highly species-specific; human renin cannot cleave rodent Agt and vice versa. Thus, hypertension develops only in double Tg mice with both hRen and hAgt [304, 305] [306].

(b) Tg mice overexpressing intrarenal RAS

In addition to the circulating RAS, considerable attention has focused on local or tissue RASs. Among these, the intrarenal RAS is of special interest. To

study the role(s) of the intrarenal RAS, Tg animal models overexpressing intrarenal RAS genes have been created. To achieve kidney-specific expression, the kidney androgen-regulated protein (KAP) promoter, which has the capacity to direct the expression of transgenes distinctively in kidney PTs, has been used extensively.

The KAP was originally identified as an abundant 20-kD polypeptide product of unknown function, derived from the *in vitro* translation of mouse kidney RNA. The KAP gene is a very abundant androgen-regulated mRNA (i.e., 4% of total mRNA) in the kidneys [307]. Serial analysis of gene expression have further revealed that KAP is the second-most abundant mRNA in the kidney [308]. *In situ* hybridization studies specifically localized KAP mRNA in proximal convoluted tubular cells of the renal cortex in normal male and testosterone-induced female mice [309]. Based on the cellular localization of KAP mRNA in the kidneys and its induction by androgen, Ding et al. developed Tg mice containing a KAP-hAgt transgene and demonstrated that the KAP promoter would specifically drive hAgt gene expression in RPTCs [310].

Based on this observation, Lavoie et al. [311] generated Tg mice expressing both human renin and Agt specifically in RPTs with a modified vector, KAP2, which was identical to the KAP-hAgt construct described above with slight modifications. In detail, the KAP-hAgt construct was modified to allow for a *Not*I insertion site downstream of the KAP promoter sequence while simultaneously deleting the entire coding potential of hAgt contained within exon II. Therefore, the construct contained the KAP promoter and noncoding DNA encompassing exons 3–5 of hAgt. The exons 3–5 of hAgt were necessary because evidence has suggested that this KAP promoter may require enhancer elements that are present downstream of the hAgt [312]. So Exons 3–5 of hAgt, in the context of this construct, are not encoded into protein, and only supply distal enhancer and exon-intron splice sites. The unique Not-1 restriction site provides feasibility for the insertion of any cDNA. Replacement of the Agt coding region with any cDNA would result in PT-specific and androgen-regulated exogenous gene expression.

Lavoie et al. observed increased BP in double Tg mice carrying both the pKAP2-hRen and KAP-hAgt transgenes. These mice were created by cross-breeding heterozygous pKAP2-hRen with heterozygous KAP-hAgt Tg mice. Biochemical and physiological studies confirmed that there was no secretion of hAgt into the systemic circulation and plasma AngII was not elevated, indicating that the mechanism of increased SBP was the result of locally-generated AngII in the kidneys. Therefore, the intrarenal RAS can have a significant effect on BP [311].

However, since the primary goal of these Tg models was to determine the effects of the RAS (or intrarenal RAS) on BP, kidney morphology and function were not investigated in detail.

(c) Tg mice used in the present study

Since rat Agt (rAgt) can be cleaved by mouse renin [313], our laboratory created Tg mice overexpressing the rAgt gene in RPTCs, with the KAP2 vector (Figure 1-28).

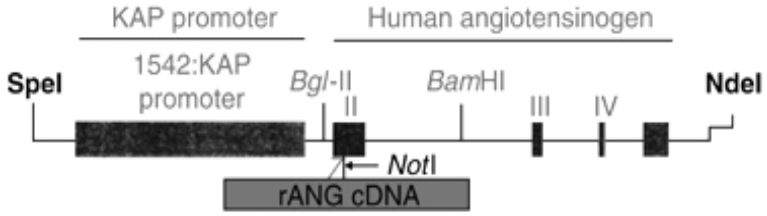

Figure 1-28. Schematic map of the kidney androgen-regulated promoter (KAP2)-rat angiotensinogen (rAgt) construct. The rAgt gene was inserted into the NotI sites in exon II. The transgene was excised as a SpeI and NdeI fragment for microinjection.

rAgt cDNA was inserted into the *Not*1 restriction site of the construct in exon II. KAP2-rAgt Tg mice were able to produce RPTC-specific and androgen-inducible rAgt in RPTCs. Southern blot analysis revealed the presence of the transgene specifically in the kidneys of heterozygote and homozygote animals [314]. Transgene expression in the kidneys was also confirmed by immunohistochemistry [251]. Therefore, the rAgt transgene expressed in RPTCs could be cleaved by renin generated from RPTCs to form AngI, and AngI would be further processed by ACE1 to form AngII. With this model, only 1 transgene would activate the intrarenal RAS.

1.6.3 *In vitro* models of intrarenal RAS activation

In vitro studies were performed to further investigate the mechanism(s) of intrarenal RAS activation. Several PTC lines were developed for experimentation, such as LLC-PK1 cells from pig tubular epithelia, HK-2 cells from human PTs, opossum kidney cortex PT epithelial cells, vEPT cells from rabbit proximal tubular epithelia, MCT cells from mouse PTs, etc. While these cell lines have been invaluable for many investigations, their use is somewhat limited because the tubular segments of origin for some cell lines are unknown, or the cell lines exhibit characteristics of more than 1 tubular segment.

1.6.3.1 Characteristics of LLC-PK1 cells

LLC-PK1 cells are generally considered to be a model for the PTC type because they possess the characteristics of PTs, such as sodium-glucose co-transport, and the presence of enzymes located in the apical membrane that includes alkaline phosphatase and γ-glutamyl transpeptidase. Nielsen et al. confirmed the expression and localization of megalin and an ability to internalize and degrade megalin-binding protein, receptor-associated protein (RAP), in the cell line. They concluded that LLC-PK1 cells were an excellent instrument for *in vitro* studies of the transcellular transport of proteins and protein-bound molecules in PTs. In addition, AT1R and ACE1 are expressed on LLC-PK1 cells which react to AngII stimulation [315] [316]. It is noteworthy that their electrolyte and fluid transport is also regulated by salmon calcitonin and antidiuretic hormone (ADH), which are characteristics of distal nephron segments [317] [318], suggesting that the LLC-PK1 cell line may not be homogeneous.

1.6.3.2 Characteristics of HK-2 cells

The HK-2 cell line was developed by transduction with human papilloma virus (HPV 16) E6/E7 genes in primary PTCs from a normal adult human renal cortex. The cells retained a phenotype of well-differentiated PTCs, and, as such, were positive for alkaline phosphatase, cytokeratin, $\alpha_3\beta_1$ integrin, and

fibronectin. Furthermore, HK-2 cells retained the functional characteristics of PTCs; for example, they exhibited Na^+ dependent/phlorizin-sensitive sugar transport and adenylate cyclase responsiveness to the parathyroids. Therefore, the generators of the cell line concluded that HK-2 cells could be a powerful new tool for the study of PTC physiology/pathophysiology and mechanisms of cell injury and repair [319]. Moreover, HK-2 cells have been used to examine the effects of the RAS [320] [321].

1.6.3.3 The vEPT cells

The vEPT cell line has been generated from the early PT (Sl segment) of rabbit kidneys and immortalized by a recombinant retrovirus encoding the SV40 large-T antigen [322]. These cells resemble primary cultures in terms of morphological, biochemical, and functional criteria. They also retain receptor and signaling mechanisms for AngII [322] [323]. Recently, Li et al. [324] confirmed a sensitive reaction of vEPT cells to AngII. Therefore, vEPT cells would be a good model of the RAS [322] [323].

1.6.3.4 IRPTCs in the present study

In our experiments, rat IRPTCs were tested as *in vitro* models. They were obtained from Dr. Julie R. Ingelfinger (Massachussetts General Hospital, Boston, MA, USA), and generated by transfecting rat PTCs, isolated from a male Wistar rat (4-5 weeks old), with temperature-sensitive SV40 mutant viruses (tsA SV40) by direct exposure [325]. TIRPTCs express a relatively large number of PT markers, such as GLUT2, carbonic anhydrase, etc. Ingelfinger et al. reported that IRPTCs express the mRNA and protein of Agt, ACE1 and AngII receptors [325] [326] [327].

Previous experiments in our laboratory demonstrated that Agt protein is secreted from IRPTCs, as measured by a specific radioimmunoassay for rAgt (RIA-rAgt), providing evidence that AngII is derived from Agt synthesized within RPTCs *in vitro* [328]. These studies support the notion that the intrarenal RAS is present in RPTs and indicate that IRPTCs are ideal for investigating the role(s)

of the intrarenal RAS in gene expression and cell function in vitro. In our experiments, IRPTCs were stably transfected with pRSV/rAgt to mimic an in vivo situation with overexpression of intrarenal RAS. IRPTCs stably transfected with the empty vector served as controls.

1.6.4 BP measurement

BP is an important clinical parameter in experimental models of hypertension, and a wide variety of techniques have been employed to measure it. The most common method of monitoring conscious animals is the use of tethers or restraining devices with measurements taken via indwelling, exteriorized vascular catheters or BP cuffs [329]. Tail-cuff measurements are widespread in basic research because of their simplicity, convenience, and capacity for simultaneous multiple recordings. The disadvantage of tail-cuff measurements, however, is that they require both warming and restraint, producing several stressful stimuli. Plasma catecholamines and cortisol are found to be elevated in these experimental animals [330]. Although tail-cuff measurement is not a perfect method, it is widely accepted to compare the BP of different groups of animals receiving the same manipulation. Indeed, after a 1-week training period, the results should be consistent in each animal.

Finally, radiotelemetry provides an alternative means of measuring BP from freely-moving, conscious animals. The Dataquest IV telemetry system consists of a transmitter, receiver panel, consolidation matrix, and personal computer with accompanying software. After calibration, a catheter for the transmitter is implanted against blood flow in the abdominal aorta of anaesthetized animals (mice or rats) just below the renal arteries. The transmitter is then sutured to the abdominal wall. After the operation, the animals are housed in individual cages placed over the receiver panel that is connected to a personal computer for data acquisition. The animals are unrestrained and free to move within their cages. BP stabilizes several days later, and telemetry data can be collected [331]. Telemetry makes it possible to gather data around the clock without stress from human contact [332]. This system is an ideal substitute for tail-cuff measurement. One of its major disadvantages, however, is its cost ($3,000 per

unit), which limits its popularity.

1.6.5 Intrarenal RAS and DN

Under diabetic conditions, measurements of RAS components in plasma indicate its suppression in animals. Morever, evidence from human studies suggests intrarenal RAS activation in DN. ACE inhibitors and AT1R blockade show potent beneficial effects by improving proteinuria in both experimental and human DN, implying intrarenal RAS activation. Furthermore, a number of research groups have demonstrated that intrarenal renin expression increased at both the mRNA and protein levels. Zimpelmann et al. reported increased renin mRNA expression in PTs from STZ-induced diabetic rats, which was completely prevented by insulin treatment, suggesting intrarenal RAS activation by hyperglycaemia [333]. Our laboratory showed that rat IRPTCs cultured in high-glucose medium expressed higher Agt levels in a dose-dependent manner [334]. Another group reported that glomerular RAS may also be activated in cultured rat mesangial cells under high intraglomerular pressure that occurs in early DN [273]. Conceivably, activation of the local intrarenal RAS in diabetes could lead to increased AngII generation in the kidneys.

1.6.5.1 Haemodynamic effects of AngII

In the early 1970s, direct micropuncture measurements in rats infused with AngII demonstrated that it heightened both efferent and afferent arteriolar resistance, but had a greater effect on efferent than on afferent arterioles. Consequently, Ang II was shown to augment glomerular capillary pressure [335] [336]. Later, in the 1980s, glomerular capillary hypertension, proteinuria and structural renal abnormalities were discovered in diabetic rats. Treatment with ACE inhibitors normalized glomerular capillary pressure and prevented the development of proteinuria and structural abnormalities, suggesting that the haemodynamic effects of AngII are critical in the pathogenesis of DN [337] [338]. Consistent with animal experiments, in the late 1990s, T2D patients with albuminuria were found to have increased glomerular pressure and efferent

arteriolar resistance compared to normoalbuminuric patients [339]. To summarize, the haemodynamic effects of AngII are crucial in the pathogenesis of DN.

1.6.5.2 Non-haemodynamic effects of AngII

A number of investigators have demonstrated that AngII can function as a growth factor for renal cells, inducing both hypertrophy and proliferation [340]. Mesangial cell proliferation results in increased ECM synthesis, contributing to glomerulosclerosis, a hallmark of DN. As discussed previously, TGF-β1 is a major contributor to diabetic kidney injury. AngII induces TGF-β1 expression, possibly through the PKC and p38 MAPK pathways and/or the JAK/STAT pathway [341] [342].

Another growth factor, vascular endothelial growth factor (VEGF) is abundantly expressed in the renal glomerular epithelia and collecting duct cells [343]. Williams et al reported that AngII can stimulate VEGF expression [344], and elevated plasma VEGF levels were demonstrated by Hovind et al. in T1D patients with DN [345]. VEGF induces angiogenesis and markedly increases microvascular permeability, indicating a key role in the pathogenesis of diabetic microvascular complications [344].

AngII has also been shown to increase PAI-1 in cultured mesangial cells, which controls plasminogen activators, generating plasmin from plasminogen. This increase in plasmin results in matrix accumulation [346].

In our laboratory, we used Tg mice overexpressing Agt in RPTCs to mimic intrarenal RAS activation. These Tg mice exhibited higher TGF-β1 and PAI-1 expression at both the mRNA and protein levels in RPTs. Furthermore, interstitial fibrosis was observed in Tg mice, providing in vivo evidence in support of AngII's hypothesized non-haemodynamic effects.

1.6.6 Clinical trials of RAS blockade in diabetic patients

Microalbuminuria is an early marker of DN, and its prevention is considered to be the key for the primary prevention of DN progression. Indeed,

the Heart Outcomes Prevention Evaluation (HOPE) Study [347], which was conducted between 1994 and 1999 with a median 4.5 years of follow-up, indicated that any degree of albuminuria was a risk factor for cardiovascular diseases in individuals with or without diabetes. According to another trial, by Mogensen et al., microalbuminuria is associated with rising BP and hyperfiltration [348]. Numerous studies have confirmed that antihypertensive treatments have renoprotective effects on microalbuminuria in T1D, although the choice of drugs is controversial. Several years ago, a meta-analysis concluded that in normotensive patients with T1D and microalbuminuria, ACE inhibitors significantly reduced progression to macroalbuminuria and increased chances of regression. Although the capacity of ACE inhibitors to change BP cannot entirely explain their antiproteinuric effect [349], accumulating data show that they prevent the progression of incipient nephropathy to overt nephropathy and preserve kidney function during long-term treatment. Therefore, ACE inhibitors are suggested as first-line therapy for all T1D patients with microalbuminuria, regardless of BP.

In T2D, several studies have also reported renoprotective effects of antihypertensive treatment in patients with hypertension and microalbuminuria [350] [351] [352], but it is still unclear whether there is a specific renoprotective effect of these drugs besides their hypotensive action. Recently, the results of a prospective, randomized, double-blind, parallel-group study, the Bergamo Nephrologic Diabetes Complications Trial (BENEDICT), showed that ACE inhibition can prevent the progression to microalbuminuria in T2D patients with hypertension and normoalbuminuria [353]. On the other hand, several clinical investigations disclosed that patients with hypertension or other risk factors for cardiovascular diseases treated with ACE inhibitors or angiotensin receptor blockers (ARBs) had a decreased risk of new-onset diabetes. Both treatments are also known to exert positive metabolic effects, such as improved insulin sensitivity [354].

Even if DN develops in T1D patients, antihypertensive treatment with ACE inhibitors still shows a renoprotective outcome: ACE inhibitors slow the

GFR decline and reduce proteinuria [355]. ARBs have similar effects on hypertensive T1D patients with DN [356]. During the last several years, 2 large multinational, placebo-controlled trials, RENAAL and IDNT, investigated the long-term renoprotective influence of ARBs in hypertensive T2D patients with DN and elevated serum creatinine. Both clinical trials demonstrated renoprotective outcomes of treatment with ARBs in the progression of DN in addition to lowering BP in T2D [357] [358]. Recently, a report from the ADVANCE clinical trial, involving 215 collaborating centres from 20 countries, noted that the routine administration of a fixed combination of the ACE inhibitor perindopril and the diuretic indapamide, to a broad range of patients with diabetes, reduced the risk of death and major macrovascular or microvascular complications. This was observed irrespective of initial BP level or ancillary treatment with other preventive agents typically provided to diabetic patients today. The researchers concluded that the treatment was well-tolerated, needed little monitoring or titration, and was suitable in a wide range of clinical circumstances worldwide. They further predicted that if the benefits seen in ADVANCE were applied to only half the population with diabetes worldwide, more than a million deaths would be avoided over 5 years. For these reasons, there is now a case for considering a combination of perindopril and indapamide routinely for patients with T2D [359].

1.7 Relationship between nephropathy and hypertension

1.7.1 Key role of the kidneys in BP regulation

Both experimental and clinical studies indicate a role of the kidneys in BP control. Several lines of evidence suggest that the kidneys are important in the genesis of primary hypertension in humans and genetic hypertension in animals. Transplantation experiments in animals and humans showed that recipients would have higher BP if donors had hypertension or a predisposition to hypertension. In early 1975, Dahl and Heine [360] conducted landmark experiments on kidney cross-transplantation between strains of hypertension-sensitive and hypertension-resistant rats. They found that the hypertension

phenotype followed the kidney; a kidney graft from a Dahl-sensitive rat caused hypertension in resistant recipients, whereas a kidney from a hypertension-resistant rat lowered BP in a sensitive recipient. Similarly, in human kidney transplantation, BP normalization was observed after transplantation of “normotensive” kidneys into hypertensive recipients [361]. These studies support the concept that many forms of essential hypertension involve a primary renal disorder that may be responsible for its induction [277].

BP is regulated by a variety of hormonal systems, including the RAS. Even in non-hypertensive conditions, intrarenal AngII levels are much higher than plasma AngII concentrations [362]. In hypertension, intrarenal AngII levels are further increased by multiple mechanisms. Moreover, the experiments conducted by Crowley et al. [363] demonstrated that AT1R in the kidneys were primarily responsible for the actions of AngII in causing hypertension. This was clearly illustrated in systemic AT1RA (a homologue of AT1R) KO animals, where the presence of AT1RA only in the kidneys was sufficient to recapitulate the phenotype of hypertension with AngII infusion. Conversely, in the kidneys of AT1RA KO mice, the absence of AT1RA from the kidneys alone was sufficient to protect them from AngII-dependent hypertension, despite AT1RA expression in a number of other key areas that potentially impact BP homeostasis, including the brain, heart, peripheral vasculature, and adrenal glands. Before these experiments, the same group [364] already reported that kidney-specific AT1RA KO mice had 20 mmHg lower BP despite normal AT1RA expression in all other tissues. In addition, they found that aldosterone levels were not affected in these KO animals. These data indicate that BP is regulated by AT1R on kidney cells. The BP reduction in kidney-specific AT1RA KO mice was a direct consequence of interrupting AT1R actions in the renal vasculature; dysfunction of the renal epithelia could influence renal sodium reabsorption, and urinary sodium excretion may contribute as well [364]. By feeding kidney-specific AT1RA KO mice a high-salt diet, this group also showed that the reduction in BP was reversed [365].

1.7.2 Hypertension causing nephropathy

The kidneys are pivotal in regulating BP. Chronic high BP, however, can cause kidney damage, which is termed hypertensive nephropathy or hypertensive nephrosclerosis. After DN, hypertensive nephrosclerosis constitutes the second largest cause of ESRD. Hypertensive nephrosclerosis affects medium- and small-sized blood vessels up to the interlobular arteries. On the other hand, the afferent and/or efferent glomerular arterioles present arteriosclerosis. The arcuate and interlobular arteries manifest myointimal hypertrophy, replication of the internal elastic lamina and hypertrophy of the media. The arterial/arteriolar wall is also thickened by hyaline, eosinophilic, Periodic-acid-Schiff-positive deposits. In these places, the smooth muscle cells are atrophic. Along with arterial changes, the glomeruli are usally ischaemic, with thickened and folded capillary walls. Typical lesions of focal segmental glomerulosclerosis are also common. The renal tubules are atrophic and microcystic, and many tubules contain proteinaceous casts. The tubulointerstitium is extensively fibrotic and hosts numerous inflammatory cells [366]. However, the relationship between hypertension and the kidneys is far from clear. One reason for this is that all the vascular and glomerular lesions described in hypertensive nephropathy can be found in normotensive, aging kidneys as well, to a greater or lesser degree. It appears that racial differences further compound the problem [367] [368]. Nonetheless, clear knowledge that obesity, smoking, carbohydrate intolerance, hyperlipidaemia, and nephrotoxin exposure all contribute to hypertensive nephrosclerosis provides evidence that this disease is treatable to a certain degree by habit changes and lowering BP to optimal levels [369].

1.8 Animal models of DN research

Because of obvious limits, it is impossible to delineate the pathogenesis of diseases in human beings. Animal models, on the other hand, are widely used in biomedical research to imitate human diseases for purposes of elucidating the causes and mechanisms of disease development as well as to

test the effects of newly-developed drugs. Several diabetic animal models of both T1D and T2D have been established. Rodents such as rats and mice are generally used to produce diabetes by various methods. STZ has been commonly employed to induce T1D, based on its ability to selectively destroy pancreatic β-cells [370]. Various doses of STZ are given to rats or mice, either intraperitoneally or intravenously, to elicit diabetes, although rat islet cells are much more sensitive to the toxic effects of STZ. However, tissue toxicity, including renal toxicity caused by STZ, is inevitable even though different doses of STZ have been tested. Tay et al. [371] reported that acute tubular necrosis, impaired GFR, and inflammatory cell infiltrate, etc. in the kidney with different doses of STZ by intraperitoneal injection were superimposed on pathological changes in diabetic kidneys. Thus, data from STZ-induced diabetes should be analyzed very carefully. Another model, the C57BL/6-*Ins2*^{Akita}/J (Akita) mouse, is becoming prevalent for research on T1D. These mice carry a mutation at the *Ins2* locus, leading to immature insulin production and resulting in spontaneous hyperglycaemia at age 3-4 weeks [372]. Islets from Akita mice are depleted of β-cells, and the remaining β-cells release very little mature insulin. Nonetheless, the severity of DN in Akita mice does not seem to be robust. Still another T1D animal model is the non-obese diabetic (NOD) mouse. Insulin is indispensable for NOD mice after the onset of hyperglycaemia. However, the genetics of the NOD model are complex. There have been relatively few studies of kidney disease in the NOD line until now.

For T2D, mice with the db/db mutation on the C57BL/6 background have been investigated intensively. Furthermore, db/db mouse show many characteristics similar to human DN. Ob/ob mice are another model of T2D. C57BL/6 mice given a high-fat diet to induce obesity and insulin resistance also serve as a T2D model. Nevertheless, the high-fat diet model and the ob/ob model develop only mild renal damage or unreported renal damage (Table 1-5) [373].

Table 1-5. Select mouse models of diabetes studied for DN (ref. [373])

Mouse model	Diabetes type	Advantages	disadvantages
Streptozotoc-in	Type 1	Well-established, reproducible, timed; may be established in strains both resistant and susceptible to DN	Potential for nonspecific toxicity; strain-dependent dosing necessary; biohazard: potential mutagen
Ins2 Akita	Type 1	Commercially available (JAX); autosomal dominant mutation	Presently only C57BL/6 commercially available; C57BL/6 relatively resistant to nephropathy; hyperglycemia in females is mild
NOD	Type 1	Spontaneous development of β-cell failure may mimic pathophysiology of the disease in humans (99); available commercially	Unpredictable timing of diabetes development; no appropriate control strain; needs insulin therapy to survive long periods; multigenetic cause of diabetes precludes easy intercrosses
Db/db	Type 2	Available on multiple strains; available commercially	Infertile; autosomal recessive; mutation in leptin receptor is a very rare cause of obesity and T2D in humans
Ob/ob	Type 2	Exists in diverse inbred strains; available commercially	Infertility-can be circumvented with leptin; autosomal recessive; nephropathy uncharacterized; mutation of leptin is a very rare cause of obesity and type 2 diabetes in humans
High-fat diet	Type 2	Onset can be determined by the investigator	Only C57BL/6 reported to be susceptible; hyperglycemia not prominent

1.9 Brief description of apocynin and hydralazine

In the present study, apocynin (an NADPH oxidase inhibitor) was administered to rAgt-Tg mice to prevent oxidative stress or ROS generation. First described by Schmiedeberg in 1883, apocynin (4-hydroxy-3-methoxyacetophenone) was isolated from the roots of *Apocynum cannabinum*

(Canadian hemp). Extracts were administered as remedies for dropsy and heart problems [374]. Apocynin was characterized as an NADPH oxidase inhibitor in the early 1980s [375]. It has been found to inhibit NADPH oxidase in many experimental models involving phagocytic and non-phagocytic cells [376] [377]. The mechanism of its inhibition is not totally known, but involves the impairment of the translocation to the membrane of the cytosolic component p47phox of the NADPH-oxidase complex [378, 379]. However, it has been noted that myeloperoxidase (MPO) is necessary for apocynin activation [375]. Presumably, apocynin is activated by H_2O_2 and MPO, forming an apocynin dimer, which then oxidizes thiols in NADPH oxidase. Apocynin does not inhibit the oxidase in cells devoid or deficient in MPO [380], and agents, such as zymosan, that promote MPO release, enhance apocynin efficacy. Moreover, it has been demonstrated that apocynin dimer is the active inhibitory compound that blocks NADPH oxidase activity but not the monomer [381].

Hydralazine has been used clinically as an anti-hypertensive agent. It is primarily vasodilatory at micromolar concentrations in the arteries and arterioles, relaxing a number of arterial preparations *in vitro*, including the rabbit aorta [382] [383] and renal artery [384] , etc. It continues to relax vessels after removal of the endothelial cell layer [383] [385], implying that it directly impacts vascular smooth muscle. The mechanism by which hydralazine relaxes vascular smooth muscles is not well-known. A directly-acting vasodilator, hydralazine has been proposed to target an intracellular site in vascular smooth muscle, inhibiting Ca^{2+} release. According to Ellershaw et al. [386], hydralazine interacts primarily with the inositol 1,4,5 trisphosphate receptor or channel to inhibit sarcoplasmic reticulum Ca^{2+} release, resulting in arterial and arteriolar relaxation. Because of its common side-effects in humans, such as headache, anorexia, and nausea, hydralazine is not a primary therapy of hypertension. In the present study, it was administered to compare its impact on hypertension and renal injury to that of perindopril and apocynin.

1.10 Objectives of the present study

Rationale: DN is the leading cause of all ESRD in North America. Both hyperglycaemia and RAS play critical roles in the progression of DN. Although the current view of DN is glomerulocentric, the gradual decline of renal function in later stages of DN is invariably associated with tubular atrophy and tubulointerstitial fibrosis. Indeed, studies have shown that tubular atrophy and interstitial fibrosis better predict renal disease progression than glomerular pathology [387] [388] [389]. However, the mechanism through which this occurs remains incompletely elucidated. Our laboratory has demonstrated that Tg mice overexpressing rAgt in their RPTCs develop hypertension and renal injury. This Tg model is characterized by intrarenal RAS activation in the absence of the circulating RAS or other tissue RAS activation. RAS blockade reverses these effects. Therefore, the objectives of the present experiments are to investigate:

1) Whether activation of intrarenal RAS could act in concert with high glucose to enhance RPTC apoptosis, and whether treatment with insulin, RAS blockers, or a combination of both could reverse these events

2) Whether ROS generation mediates, at least in part, the pro-apoptotic and pro-fibrotic effects of intrarenal RAS activation in PTs in vivo and whether inhibition of NADPH oxidase could prevent RPTC apoptosis, independently of systemic hypertension

3) Whether ER stress may be involved in RPTC apoptosis in Akita Tg mice overexpressing rAgt in RPTCs and to explore the effects of RAS blockade on ER stress.

Chapter 2: Article 1

Enhanced Tubular Apoptosis in Diabetic Transgenic Mice Overexpressing Angiotensinogen Gene

Enhanced Tubular Apoptosis in Diabetic Transgenic Mice Overexpressing Angiotensinogen Gene

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2.1 Abstract

We investigated whether activation of the intrarenal renin-angiotensin system (RAS) could contribute to renal proximal tubular cell (RPTC) apoptosis in diabetes. Adult male transgenic (Tg) mice overexpressing rat angiotensinogen (rAgt) gene in their RPTCs and similarly aged non-Tg littermates were each randomly divided into 4 groups: 1. non-diabetic controls; 2. streptozotocin (STZ)-induced diabetes; 3. insulin-treated diabetes; and 4. RAS blockers-treated diabetes. Diabetic Tg mice treated with RAS blockers and insulin as well as Tg mice treated with hydralazine were also studied. Animals were euthanized after 4 weeks of diabetes. Renal histology and apoptosis studies were performed. Bax and Bcl-xL mRNA levels were quantified by real-time quantitative PCR assays. Rat immortalized RPTCs stably transfected with Agt cDNA or control plasmid were cultured in normal or high glucose medium +/- insulin and/or RAS blockers. Diabetic rAgt-Tg mice developed significantly higher blood pressure and augmented albuminuria, RPTC apoptosis, and pro-apoptotic gene expression as compared to diabetic non-Tg littermates. Insulin and/or RAS blockers markedly attenuated these changes. Hydralazine treatment prevented hypertension but not albuminuria, RPTC apoptosis and pro-apoptotic gene expression. *In vitro* studies revealed that high glucose significantly enhances apoptosis and caspase-3 activity in Agt-stable transfectants compared to control cells. These changes were prevented in the presence of insulin and/or RAS blockers. These studies suggest that intrarenal RAS activation and high glucose may act additively to enhance tubular apoptosis in diabetes independent of systemic hypertension.

2.2 Introduction

Diabetic nephropathy (DN) is the leading cause of all end-stage renal diseases (ESRD) in North America, accounting for 45-50% of all cases.[1, 2] Intensive insulin therapy and chronic treatment with renin-angiotensin system (RAS) blockers are effective in retarding DN progression, but do not provide a cure.[3-5]

The local intrarenal RAS is well accepted.[6, 7] Renal proximal tubular cells (RPTCs) express all components of the RAS.[8-10] Angiotensin II (Ang II) levels and RAS genes are elevated in the kidneys of diabetic rats and humans,[11-13] implying an important role for the intrarenal RAS in DN progression.

Glomerular changes in DN first consist of hypertrophy, and later, thickening of the glomerular basement membrane, followed by expansion of the mesangial matrix and glomerulosclerosis.[14-16] However, the gradual decline of renal function in later stages of DN is invariably associated with tubular atrophy and interstitial fibrosis, hallmarks of ESRD.[17,18] In fact, tubular atrophy and interstitial fibrosis are closely associated with loss of renal function and appear to be better predictors of renal disease progression than glomerular pathology.[15-18] Tubular atrophy in DN is incompletely understood, though apoptosis is a candidate mechanism. Indeed, apoptosis has been detected in RPTCs of diabetic mouse, rat and human kidneys.[19-24]

Our previous study documented that transgenic (Tg) mice overexpressing rat Agt (rAgt) cDNA in RPTCs are prone to develop hypertension, albuminuria and renal injury.[25] The present study investigated whether intrarenal RAS activation could act in concert with high glucose to enhance RPTC apoptosis independent of systemic hypertension and whether treatment with insulin, RAS blockers or a combination of both could reverse these events.

2.3 Results

Tissue-specific Expression of the rAgt Transgene in Tg Mice

The rAgt-HA transgene was expressed only in the kidneys (Figure 1A) and RPTs of adult male Tg mice (Figure 1B) but not in other tissues and RPTs of non-Tg mice. Immunostaining confirmed the increased expression of Agt protein in RPTCs of Tg mice (Figure 1C). In subsequent experiments, testosterone pellets were not used because the endogenous testosterone was sufficient to induce rAgt-HA transgene in adult male Tg mice.

Renal Hypertrophy and Albuminuria in Diabetic rAgt-Tg Mice

As expected, STZ administration resulted in elevated blood glucose levels in both non-Tg and Tg mice (Figure 2A), and increased the kidney/body weight and urinary albumin/creatinine ratio (Figures 2B and 2C). Insulin normalized these parameters in both diabetic non-Tg and Tg mice. Treatment with RAS blockers also attenuated the kidney/body weight ratio and the urinary albumin/creatinine ratio without affecting blood glucose level. Treatment with insulin plus RAS blockers completely reversed all changes observed in diabetic Tg mice (Figures 2B and 2C). Of note, the urinary albumin/creatinine ratio in Tg mice was significantly higher than in non-Tg mice (Figure 2C).

Hypertension in Tg Mice

We detected slight elevations in mean systolic BP in male Tg mice starting at week 11, however, these increases became statistically significant only at week 12 and thereafter (Figure 3A). Hydralazine treatment (commenced on week 14) reduced BP of Tg mice to that of non-Tg mice after 1-week of treatment (Figure 3A). STZ-induced diabetes did not evoke further increases in BP in non-Tg and Tg mice, and insulin treatment did not reduce BP (Figure 3B). In contrast, treatment with RAS blockers alone or in combination with insulin significantly decreased BP in both diabetic non-Tg and Tg mice (Figure 3B).

Morphological Studies

As expected, diabetic non-Tg mice developed glomerular and RPTC hypertrophy (Figure 4A, a and b), and exhibited a large number of detached cells and cell debris in the tubular lumen and loss of the brush border in RPTCs in diabetic kidneys, indicating RPTC damage. Treatment with insulin (Figure 4A, c) or RAS blockers (Figure 4A, d) attenuated the glomerular hypertrophy and tubular damage in diabetic non-Tg mice.

Unlike non-Tg mice, renal structural damage was evident in non-diabetic Tg mice. Histological findings included vacuole degeneration in RPTCs and accumulation of cell debris in the tubular lumen (Figure 4A, e). Kidneys of diabetic Tg mice showed even more severe morphological changes, including

marked tubular luminal dilation, cell debris accumulation inside tubules and loss of brush border in RPTCs (Figure 4A, f). Some RPTCs even became flattened. Treatment with insulin (Figure 4A, g) or RAS blockers (Figure 4A, h) markedly attenuated, whereas combination of insulin and RAS blockers completely reversed these abnormalities (Figure 4A, i).

Glomerular volume, RPTC volume and tubular luminal area were significantly increased in diabetic non-Tg and Tg mice as compared to non-diabetic littermates (Figure 4B, C and D). Once again, treatment with insulin or RAS blockers alone effectively attenuated, whereas the combination treatment fully reversed these changes. It should be noted that RPTC volume (Figure 4C) but not glomerular volume (Figure 4B) was significantly higher in Tg mice than in non-Tg mice. In contrast, tubular luminal area was significantly lower in Tg mice than in non-Tg mice (Figure 4D). Similar pattern for cell debris quantification was observed in these studies (data not shown).

rAgt Overexpression Augments Apoptosis in Diabetic RPTCs

Apoptotic cells were detected in RPTs and in a few distal tubules (DTs) of diabetic non-Tg mice (Figure 5A, a and b). Treatment with insulin (Figure 5A, c) or RAS blockers (Figure 5A, d) significantly attenuated development of apoptosis in RPTs and DTs in diabetic non-Tg mice. The number of apoptotic RPTCs was higher in Tg mice than in non-Tg mice (Figure 5A, a and e) and was even higher in diabetic Tg mice (Figure 5A, f). While treatment with insulin (Figure 5A, g) or RAS blockers alone (Figure 5A, h) effectively attenuated, insulin plus RAS blockers fully prevented RPTC apoptosis (Figure 5A, i). Semi-quantitative estimation of the number of apoptotic RPTCs confirmed these findings (Figure 5B).

Likewise, immunohistochemistry showed increased staining for active caspase-3 and Bax in RPTCs of diabetic non-Tg mice (Figure 6A, b and Figure 7A, b) as compared to non-diabetic non-Tg mice (Figure 6A, a and Figure 7A, a). Treatment with insulin (Figure 6A, c and Figure 7A, c) or RAS blockers (Figure 6A, d and Figure 7A, d) effectively attenuated these changes. RPTCs of

Tg mice expressed active caspase-3 and Bax at higher levels (Figure 6A, e and Figure 7A, e) than non-Tg mice (Figure 6A, a and Figure 7A, a). Kidneys of diabetic Tg mice exhibited the highest levels of active caspase-3 and Bax (Figure 6A, f and Figure 7A, f) and these were effectively attenuated by insulin (Figure 6A, g and Figure 7A, g) or RAS blockade (Figure 6A, h and Figure 7A, h). Combination of insulin and RAS blockers resulted in almost complete reversal of these changes (Figure 6A, i and Figure 7A, i). Similar trends were detected by semi-quantitation of the number of caspase-3 and Bax positive RPTCs (Figure 6B) and by Western blotting of active caspase-3 and Bax from isolated RPTs (Figure 6C and 7C). Taken together, these data demonstrate that rAgt overexpression further enhances RPTC apoptosis and pro-apoptotic gene expression in diabetic Tg mice.

rAgt Overexpression Enhances Bax and Decreases Bcl-xL mRNA Expression in Mouse RPTs

Hyperglycemia alone increased Bax mRNA (Figure 8A) and decreased Bcl-xL mRNA expression (Figure 8B) in both non-Tg and Tg mouse RPTs. Combined treatment with insulin and RAS blockers were more effective in reversing these changes than treatment with insulin or RAS blockers alone. These studies demonstrate that hyperglycemia and intrarenal RAS activation act in concert to alter the ratio of Bax and Bcl-xL mRNA expression, consistent with enhanced RPTC apoptosis.

Effect of Hydralazine in Tg mice

Hydralazine treatment markedly reduced systemic BP in Tg mice without producing significant effects on blood glucose, the kidney/body weight ratio and albuminuria (Figure 9). Furthermore, hydralazine failed to affect RPTC apoptosis (Figure 10), caspase-3 and Bax immunostaining, and Bax and Bcl-xl mRNA expression (Figure 11). These results indicate that albuminuria and RPTC apoptosis occur independently of systemic hypertension.

Agt Overexpression and High Glucose Enhanced Rat Immortalized RPTC Apoptosis in vitro

Culture of RPTCs stably transfected with pRC/RSV or pRSV/rAGT in high glucose medium led to enhanced apoptosis and caspase-3 activity (Figure 12). Consistent with our in vivo observations, high glucose-induced RPTC apoptosis and caspase-3 activity were attenuated in the presence of insulin or RAS blockers with complete inhibition being achieved in the presence of both insulin and RAS blockers (Figure 12). Thus, RAS activation and high glucose can directly induce RPTC apoptosis.

2.4 Discussion

The present observations indicate that renal rAgt overexpression and high blood glucose act in concert to induce albuminuria and RPTC apoptosis in diabetic Tg mice independent of systemic hypertension.

The transgene expression in our model is kidney-specific since strong rAgt-HA mRNA and Agt protein expression were observed in RPTCs of Tg mice. 4 weeks post-STZ, the mice exhibited high blood glucose levels, increased kidney to body weight ratio and albuminuria, characteristics of STZ-induced diabetes.[26,27] Insulin treatment ameliorated these changes. Treatment with RAS blockers exerted similar beneficial actions with the exception of blood glucose level, which remained high following RAS blockade. These findings suggest that renal changes can be attributed to the diabetic state per se rather than to a nephrotoxic effect of STZ.

Male Tg mice had significantly higher systolic BP than non-Tg littermates, and STZ-induced diabetes did not lead to significant additional increases in systolic BP. The effectiveness of RAS blockers in reducing BP in Tg mice implicates the involvement of intrarenal RAS activation in the development of hypertension.

Our data indicate the presence of apoptosis in RPTCs and, to a lesser degree in the distal tubule, with no obvious involvement of glomeruli in diabetic kidneys regardless of the transgene expression. Indeed, glomeruli of diabetic Tg

and non-Tg animals did not show apoptotic morphology, and stained negative for active caspase-3 and Bax. These observations are consistent with previous findings that diabetes induces apoptosis in RPTs, but not in glomeruli of db/db mice and STZ-diabetic rats.[28,29]

Our results highlight an important role for intrarenal Agt gene expression in mediating tubular apoptosis in the diabetic mouse kidney. Indeed, the numbers of TUNEL-positive RPTCs were significantly higher in Tg mice than in non-Tg mice and were effectively reduced following treatment with RAS blockers. Losartan reduced the number of apoptotic cells in kidneys of STZ-induced diabetic rats[21] and Ren-2 rats,[22] and prevented Ang II-stimulated RPTC apoptosis[30] and glomerular cells *in vitro*.[31] Consistent with the findings in the TUNEL assay, RPTs of diabetic non-Tg and Tg mice exhibited enhanced active caspase-3 and Bax expression. These changes were attenuated by treatment of mice with insulin or RAS blockers alone, with complete inhibition being achieved with insulin plus RAS blockers. While active caspase-3 and Bax expression was undetectable in wild type animals by immunostaining and Western blotting, both caspase 3 and Bax were highly expressed in Tg mice. Enhanced Bax expression occurred with concomitant down-regulation of the anti-apoptotic gene Bcl-xL. Treatment with insulin or RAS blockers reversed the Bax/Bcl-xL ratio that was further reduced by insulin plus RAS blockers. An increase in the Bax/Bcl-xL ratio would promote apoptosis and is a likely mechanism how intrarenal RAS activation and high blood glucose enhanced tubular apoptosis in diabetes.

Next, we addressed whether intrarenal RAS-induced kidney injury was due to elevated systemic BP. Hydralazine treatment of Tg mice decreased hypertension, however, it failed to affect albuminuria, RPTC apoptosis and pro-apoptotic gene expression. This study shows for the first time that intrarenal rAgt overexpression leads to the development of hypertension, albuminuria and RPTC apoptosis. Interestingly, data suggest that albuminuria and apoptosis develop independently of systemic hypertension. Our *in vitro* findings that rAgt overexpression and high glucose can induce RPTC apoptosis further indicate a

direct action on the kidney. Taken together, these data provide evidence that intrarenal RAS and high glucose could act in concert to induce tubular apoptosis in diabetes.

The precise molecular mechanism(s) by which intrarenal RAS and high glucose enhanced RPTC apoptosis in our Tg mouse model remain unclear. High glucose and Ang II are known to stimulate ROS and ROS through activation of p38 MAPK[32] would stimulate p53 phosphorylation.[33] Following translocation to the nucleus, the phosphorylated p53 would enhance Bax gene transcription.[34] Bax translocates to the mitochondria and binds the anti-apoptotic proteins Bcl-2 and Bcl-xL, and inhibits their protective actions on gate-keeping, ultimately leading to mitochondrial dysfunction and activation of caspase-3. Our data on increased Bax and caspase-3 expression combined with decreased Bcl-xL expression in RPTs of diabetic Tg mice lend support to this notion.

The pathophysiological importance of RPTC apoptosis induced by rAgt overexpression remains to be elucidated. Since tubular cells undergo apoptosis in murine and human diabetic kidneys[19-24] and the present study, we propose that RPTC apoptosis may be an initial mechanism leading to tubular atrophy that has been detected in the kidneys of diabetic patients.[35,36]

Taken together, our results suggest that activation of intrarenal RAS and high blood glucose act in concert to induce RPTC apoptosis independent of systemic hypertension and likely lead to tubular atrophy in diabetes. Our data also indicate that treatment with insulin plus RAS blockers could prevent tubular apoptosis and DN progression.

2.5 Materials and Methods

The pKAP2 plasmid containing the kidney androgen-regulated promoter (KAP), which is responsive to testosterone stimulation, was obtained from Dr. Curt D. Sigmund (University of Iowa, Iowa City, IA, USA) and has been described elsewhere.[37,38] A rabbit polyclonal antibody against rAgt was generated in our lab (J.S.D.C.).[39] Anti-Bax and anti-active caspase-3 polyclonal antibody were procured from New England Biolabs Ltd (Pickering, ON, Canada).

Losartan (a non-peptide Ang II-receptor subtype 1 (AT_1-R) blocker) and perindopril (an inhibitor of angiotensin-converting enzyme) were obtained from Dupont Merck (Wilmington, DE, USA) and Servier Amérique (Laval, Quebec, Canada), respectively. Insulin implants (LinBit) (insulin release rate: ~0.1 unit/implant/day for >30 days) were purchased from Linshin (Scarborough, ON, Canada). Oligonucleotides were synthesized by InVitrogen, Inc. (Burlington, ON, Canada). Restriction and modifying enzymes were from either InVitrogen, Inc. or La Roche Biochemicals (Dorval, QC, Canada).

Agt Tg Mice

We created Tg mice (C57Bl6 background, line #388) by inserting rAgt cDNA fused with a HA tag (a sequence encoding amino acid residues 98-106 (YPYDVPDYA) of human influenza virus hemagglutinin) at the 3' terminal into a construct containing the KAP promoter.[25] All animals received standard mouse chow and water *ad libitum*. The experimental procedures were approved by the Animal Protection Committee of the CHUM.

Physiological Studies

Male Tg mice and non-Tg littermates were divided into 4 groups (8 mice per group). Group 1 (control group) received vehicle intraperitoneally (i.p.). Group 2: Streptozotocin (STZ)-induced diabetes. The animals were administered STZ (50 mg/kg in 10 mM sodium citrate buffer, pH 4.5), i.p., daily for 5 consecutive days. 48 h after the last STZ administration, blood glucose level was determined with a Side-Kick Glucose Analyzer (Model 1500, Interscience, ON, Canada). Mice with blood glucose >16.6 mM were studied. Group 3: Insulin treatment. On day 3 after the last STZ injection, diabetic mice received a subcutaneous insulin implant (1 Linβit implant per 20 g body weight, and a 0.5 Linβit implant for each additional 5 g body weight) to maintain euglycemia. Blood glucose was monitored twice a week. Group 4: Treatment with RAS blockers. Beginning on day 3 after the last STZ injection, mice received RAS blockers (losartan (30 mg/kg/day) plus perindopril (4 mg/kg/day)

in the drinking water). Additionally, STZ-induced diabetic Tg mice treated with RAS blockers plus insulin and Tg mice treated with hydralazine (15 mg/kg/day in drinking water), were also studied.

Systolic blood pressure (BP) was monitored with a BP-2000 tail-cuff pressure device (Visitech Systems, Apex, NC, USA)[25] in the morning, at least 3 to 4 times a week for 10 weeks after habituating mice to this procedure for at least 30-60 min per day for 5 days prior to the first BP reading. Each point represents the means ± S.D. of 3 to 4 determinations per week per group of animals.

Twenty-four h prior to euthanasia with CO_2 at 4 weeks post-STZ administration, the animals were housed individually in metabolic cages to collect urine samples later assayed for glucose and ketone levels (Keto-Diastix, Bayer Inc., Healthcare Division, Toronto, ON, Canada), and albuminuria (ELISA, Albuwell and Creatinine Companion, Exocell, Inc., Philadelphia, PA, USA).[25] Body weight and blood glucose was measured. Both kidneys were removed, decapsulated and then weighed together. Left kidneys were processed for histology and the right kidneys were used for isolation of renal proximal tubule (RPT).[25,40]

Morphological Studies

4-5 sections per kidney (8 animals per group) were counterstained with hematoxylin and eosin (H/E),[25] and analysed by two investigators unaware of the treatments.

The mean glomerular volume on 30 random glomerular sections per mouse was determined by the method of Weibel[41] with the Motics Images Plus 2.0 image analysis software (Motic, Richmond, BC, Canada). RPTC volume and tubular luminal area were measured from 100 RPTCs and 50 RPTs per mouse, respectively, with the same software. Outer cortical RPTs with similar cross-sectional views and clear nuclear structure were selected. The mean cell volume was estimated by the Nucleator method.[42]

Terminal Transferase-Mediated Deoxyuridine Triphosphate (dUTP) Nick End-labeling (TUNEL) Assay and Immunohistochemical Staining

The percentage of apoptotic RPTCs (TUNEL kit, Roche Diagnostics) was estimated semi-quantitatively as described by Kumar et al.[21,23] Immunohistochemical staining was performed by standard avidin-biotin-peroxidase complex method (ABC Staining System, Santa Cruz Biotechnologies, Santa Cruz, CA, USA).[25]

Western Blotting and Real time-Quantitative Polymerase Chain Reaction (RT-qPCR) Assays for Gene Expression

Western blotting was performed as described.[25,39]. Bax and Bcl-xL mRNA expression in mRPTs was quantified by RT-qPCR[43] using forward and reverse primers corresponding to Bax,[44] Bcl-xL,[45] and β-actin cDNA[46] (Table I).

Cell Culture and Caspase-3 Activity Assay

Rat immortalized RPTCs stably transfected with the control plasmid pRC/RSV or with a plasmid pRSV/rAgt containing the rAgt cDNA were cultured as described.[47]

Caspase-3 activity was determined fluorimetrically (BD Bioscience Pharmingen, Mississauga, ON, Canada).[48]

Statistics

Data are expressed as mean ± SD. The data are analyzed by one-way ANOVA and the Bonferroni test. P values <0.05 were considered significant.

2.6 Acknowledgements

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from the Foundation of the CHUM. The editorial assistance of Mr. Ovid M. Da Silva, Research Support Office, Research Centre, CHUM, is acknowledged.

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2.8 Legends and Figures

Table 2-I. Primers for RT-PCR

Bax (S) N+57 to N+76 (5'-TCA TGA AGA CAG GGG CCT TTT-3') Bax (AS) N+293 to N+273 (5'-CAA TCA TCC TCT GCA GCT CCA-3') (L22472)
Bcl-xL (S) N543 to N566(5'- TACCGGAGAGCGTTCAGTGATCTA-3') Bcl-xL (AS) N699to N722(5'-CTGCATCTCCTTGTCTACGCTTTC-3') (L35049)
β-actin (S) N+155 to N+179 (5'-ATG CCA TCC TGC GTC TGG ACC TGG C-3') β-actin (AS) N+115 to N+139 (5'-AGC ATT TGC GGT GCA CGA TGG AGG G-3') (NM 031144)

S, sense; AS, antisense

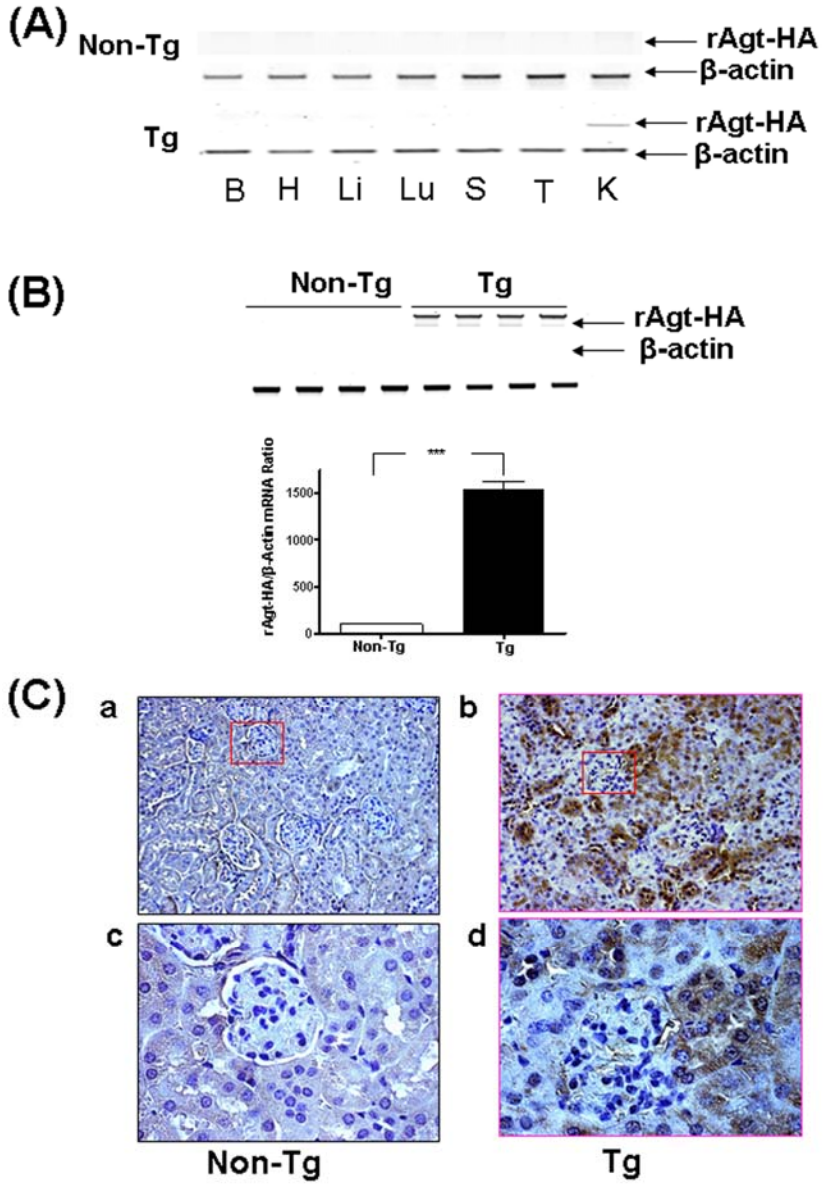

Figure 2-1. Transgene expression. A) RT-PCR product showing tissue expression of rAgt-HA mRNA in adult male non-Tg and Tg mice (18-20 weeks old). rAgt-HA and β-actin fragments are indicated: B, brain; H, heart; Li, liver; Lu, lung; S, spleen; T, testis and K, kidney;. B) Quantification of rAgt-HA mRNA level in RPTs of male non-Tg mice and Tg mice (line #388) by conventional RT-PCR. The relative densities of rAgt-HA mRNA were normalized with control β-actin mRNA. All data are expressed as means ± S.D., N=8, ***$p<0.005$). C) Immunohistochemical staining of Agt in male non-Tg (a and c) and Tg (b and d) mouse kidneys was accomplished by employing rabbit anti-rAgt (1:100 dilution) polyclonal antibody. Non-immune normal rabbit serum displayed no immunostaining for Agt in both non-Tg and Tg mouse kidneys (not shown). Note: in a and b magnification X 200; in c and d, magnification X 600).

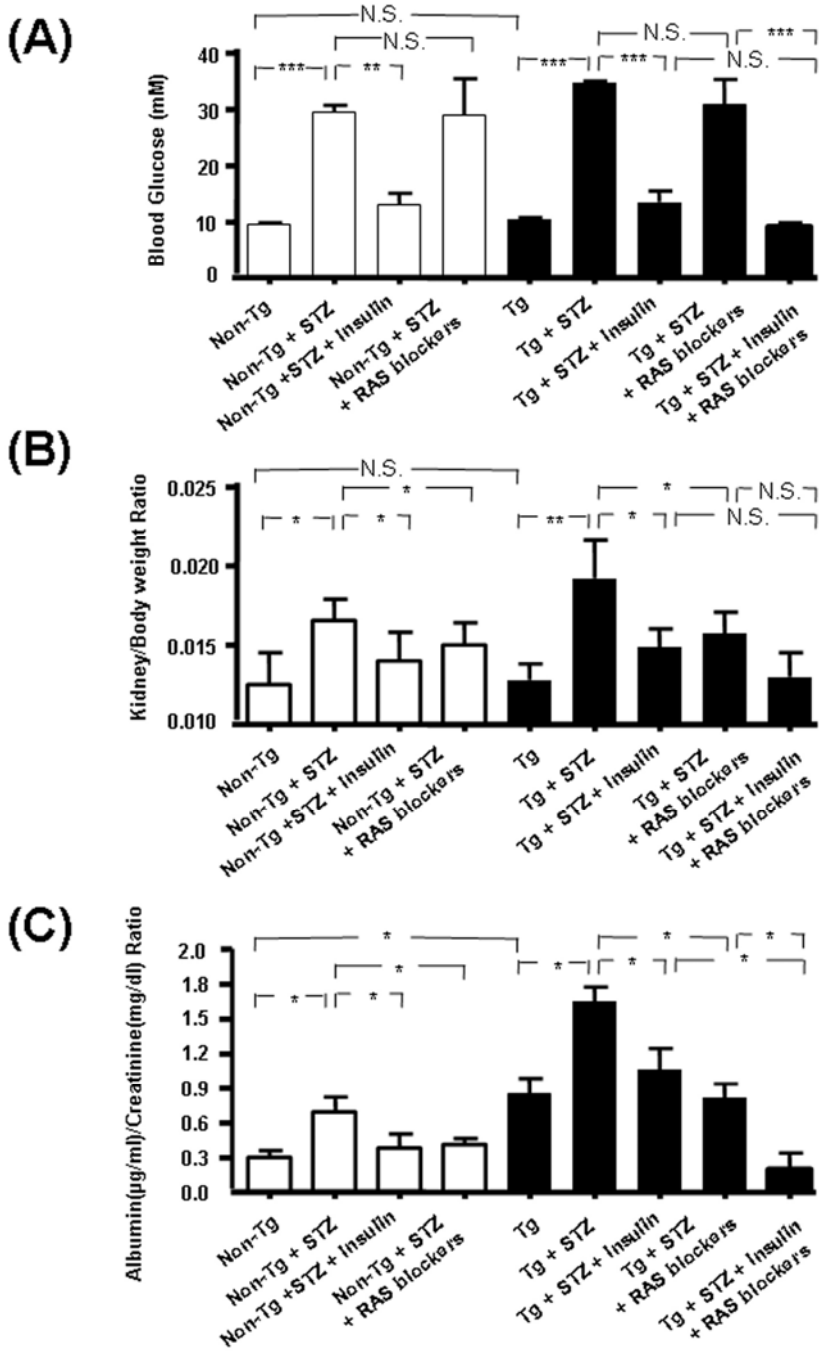

Figure 2-2. Blood glucose, kidney/body weight ratio, and urinary albumin/creatinine ratio in male non-Tg and Tg mice after 4 weeks of STZ-induced diabetes with or without treatment with insulin or RAS blockers. (A) Blood glucose. Blood glucose was measured was done in the morning without fasting at the end of the study. (B) Kidney-to-body weight ratio. The kidney-to-body weight ratio was measured as the weight of 2 kidneys per body weight. (C) Urinary albumin (μg/ml)/creatinine (mg/dL) ratio. All data are expressed as means ± SD, N=8 (*$p<0.05$, **$p<0.01$; ***$p<0.005$; N.S., not significant). Non-Tg (empty bars) and Tg (solid bars) mice.

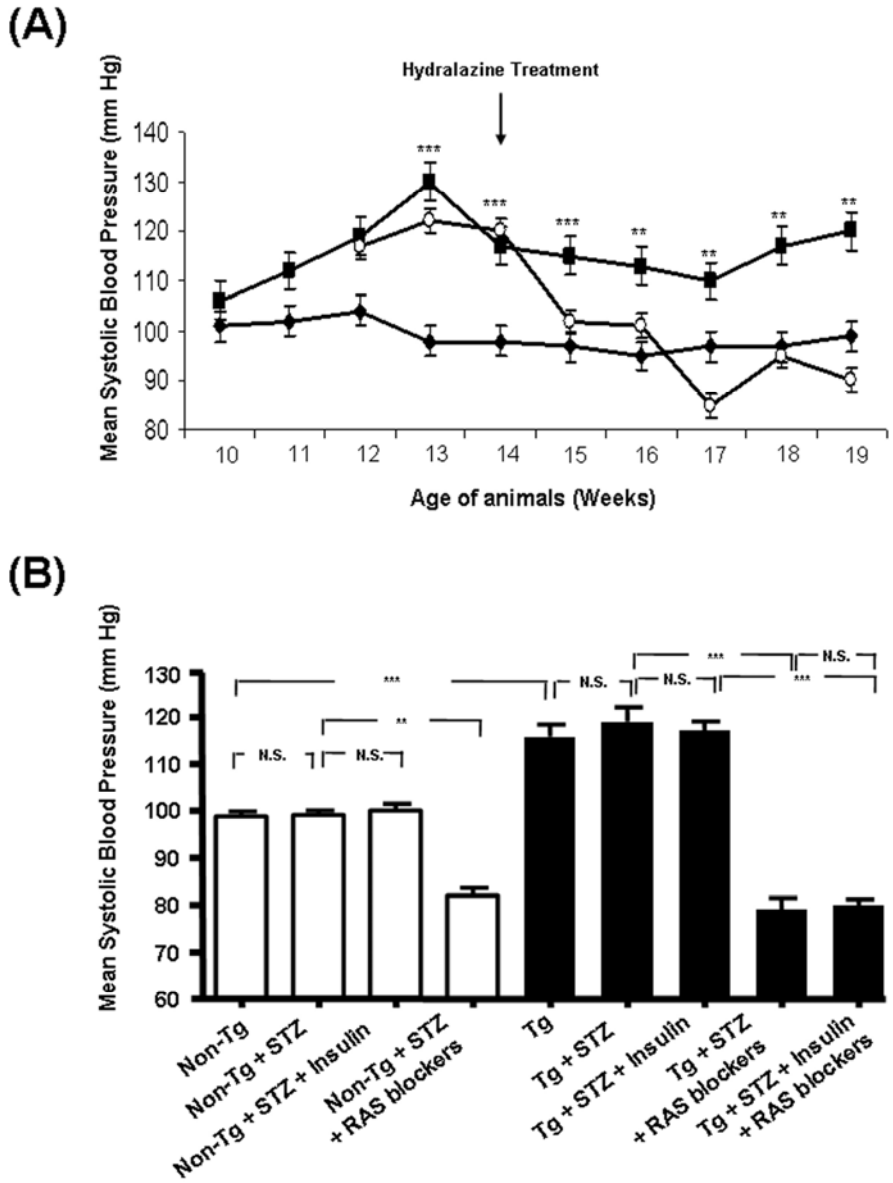

Figure 2-3. Systolic BP in Tg and non-Tg mice. A) Longitudinal changes in mean systolic BP in male non-Tg and Tg mice with or without hydralazine treatment for a 19-week period. Baseline BP was measured in all mice for 5 days before the first BP reading. Non-Tg mice (♦), Tg mice (●), and Tg mice treated with hydralazine (○) starting at week 14. B) Cross-sectional analysis of blood pressure (measured 3 to 4 times per week in the morning without fasting at the end of the study, week 20) in non-Tg and Tg mice after 4 weeks of STZ-induced diabetes with or without treatment with insulin or RAS blockers or insulin plus RAS blockers. All data are expressed as means ± SD, N=8 (**$p<0.01$; ***$p<0.005$; N.S., not significant). Non-Tg (empty bars) and Tg (solid bars) mice.

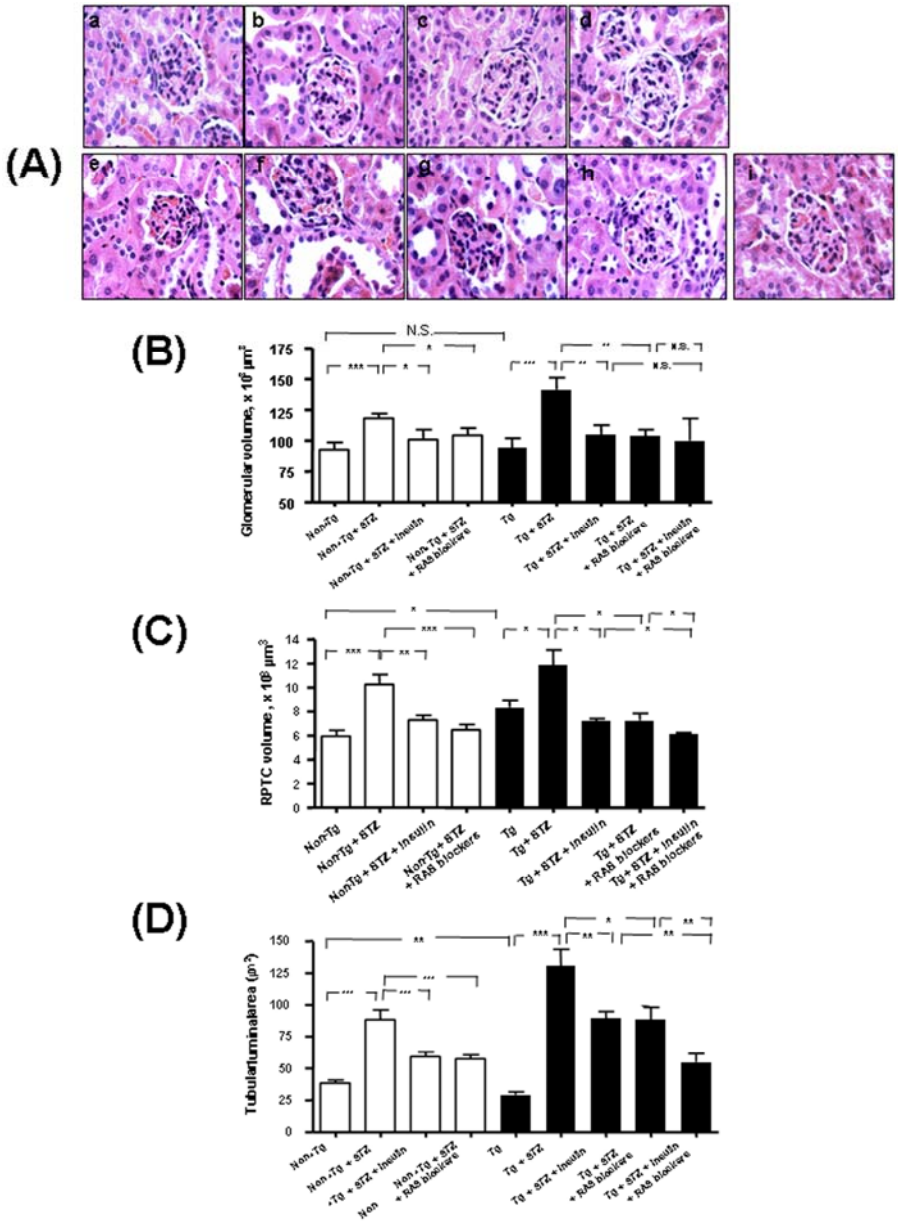

Figure 2-4. Hematoxylin/eosin staining of kidneys of male non-Tg mice and Tg mice after 4 weeks of STZ-induced diabetes with or without treatment with insulin, RAS blockers or insulin plus RAS blockers. A. a, Non-diabetic control; b, STZ-induced diabetes; c, Insulin-treated diabetic mouse; d, RAS blockers-treated diabetic mouse; e, Non-diabetic Tg mouse; f, STZ-induced diabetic Tg mice; g, Insulin-treated, STZ-induced diabetic Tg mice; h, RAS blockers treated, STZ-induced diabetic Tg mice; i, Insulin plus RAS blockers treated, STZ-induced diabetic Tg mice. Magnification, X600. B. Mean glomerular volume of male non-Tg and Tg mouse kidneys with or without treatment at week 20. C. Mean RPTC volume of male non-Tg and Tg mouse kidneys with or without treatment at week 20. D. Mean tubular lumen area of male non-Tg and Tg mouse kidneys with or without treatment at week 20. Values are expressed as means ± S.E., N=8) (*p<0.05; **p<0.01, ***p<0.005; N.S., not significant). Non-Tg (empty bars) and Tg (solid bars) mice.

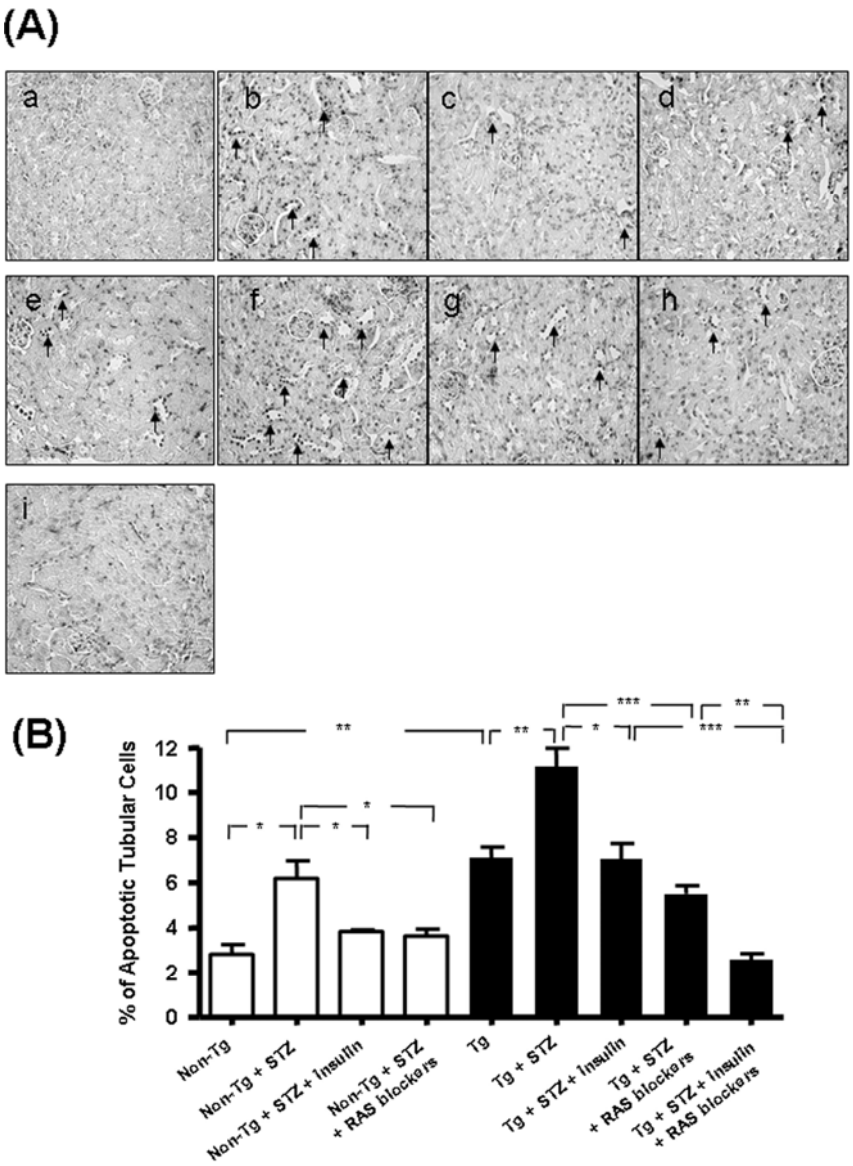

Figure 2-5. Apoptosis in male non-Tg and Tg mouse kidneys after 4 weeks of STZ-induced diabetes with or without treatment with insulin or RAS blockers or insulin plus RAS blockers, analyzed by TUNEL assay. A. a, Non-diabetic controls; b, STZ-induced diabetes; c, insulin-treated, diabetic mouse; d, RAS blockers-treated, diabetic mouse; e, non-diabetic Tg mouse; f, STZ-induced diabetic Tg mouse; g, insulin-treated, STZ-induced diabetic mouse; h, RAS blockers-treated, diabetic Tg mouse; i, Insulin plus RAS blockers-treated, diabetic Tg mouse. Magnification x200. Arrows indicate apoptotic cells in proximal tubule. B. Bar graph showing semi-quantitative analysis of apoptotic RPTCs from male non-Tg and Tg mouse kidneys after 4 weeks of STZ-induced diabetes with or without treatment with insulin or RAS blockers or insulin plus RAS blockers. All data are expressed as means ± SD, N=8 (*$p<0.05$, **$p<0.01$; ***$p<0.005$; N.S., not significant). Non-Tg (empty bars) and Tg (solid bars) mice.

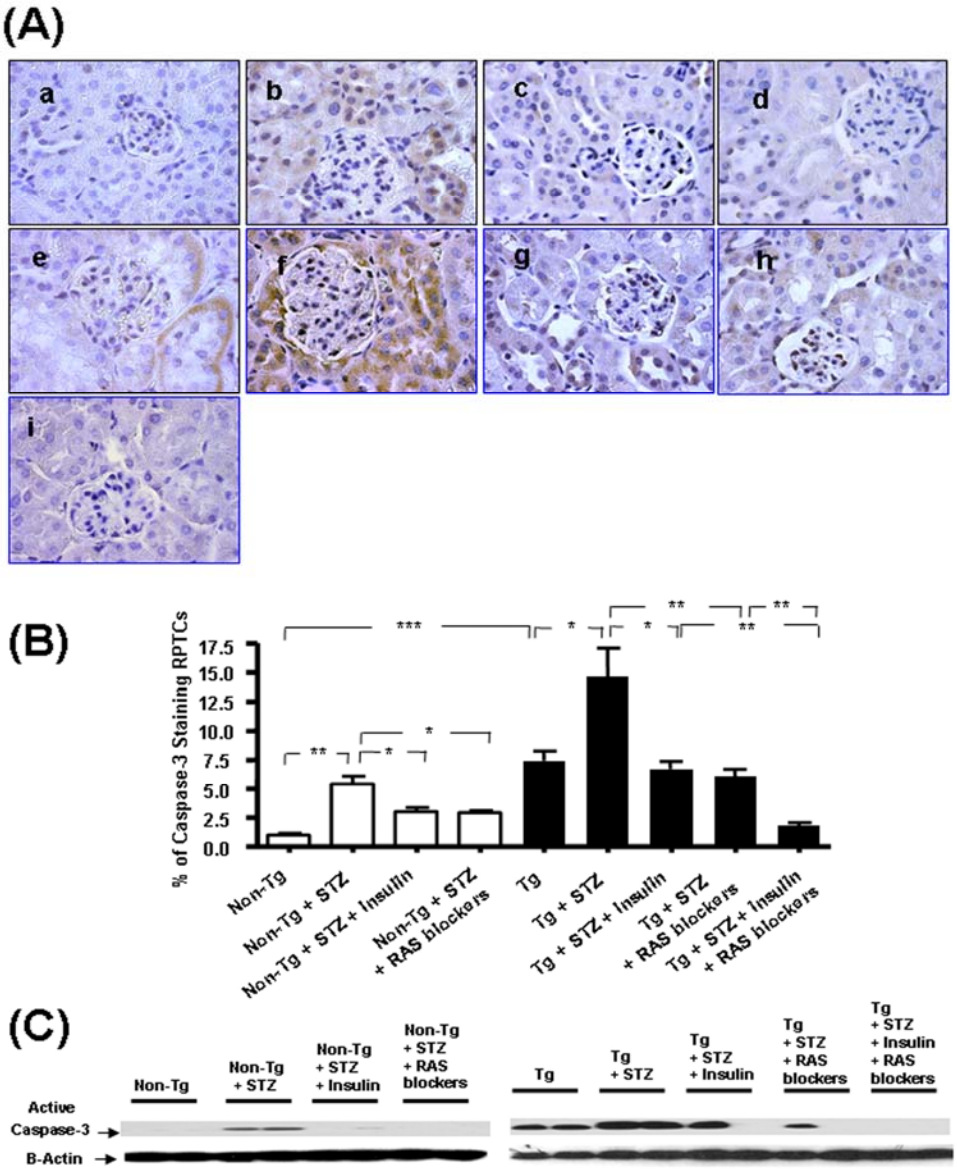

Figure 2-6. Immunohistochemical staining and Western Blots of α-active caspase-3 in male non-Tg and Tg mouse kidneys after 4 weeks of STZ-induced diabetes with or without treatment with insulin or RAS blockers or insulin plus RAS blockers employing rabbit anti-α-active caspase-3 antibodies (1:50 dilution). A. a, non-diabetic controls; b, STZ-induced diabetes; c, insulin-treated diabetic mouse; d, RAS blockers-treated diabetic mouse; e, non-diabetic Tg mouse; f, STZ-induced diabetic Tg mouse; g, insulin-treated, STZ-induced diabetic mouse; h, RAS blockers-treated, diabetic Tg mouse; i, insulin plus RAS blockers-treated, diabetic Tg mouse. Antibody dilution 1:50 and magnification x 200. B. Semi-quantitative analysis of caspase-3 staining RPTCs from male non-Tg and Tg mouse kidneys after 4 weeks of STZ-induced diabetes with or without treatment with insulin or RAS blockers or insulin plus RAS blockers. All data are expressed as means ± SD, N=8 (*$p<0.05$, **$p<0.01$; ***$p<0.005$). Non-Tg (empty bars) and Tg (solid bars) mice. C. Western blot analysis of active caspase-3 expression (antibody dilution 1: 500) in mouse RPT extracts of male non-Tg and Tg mice.

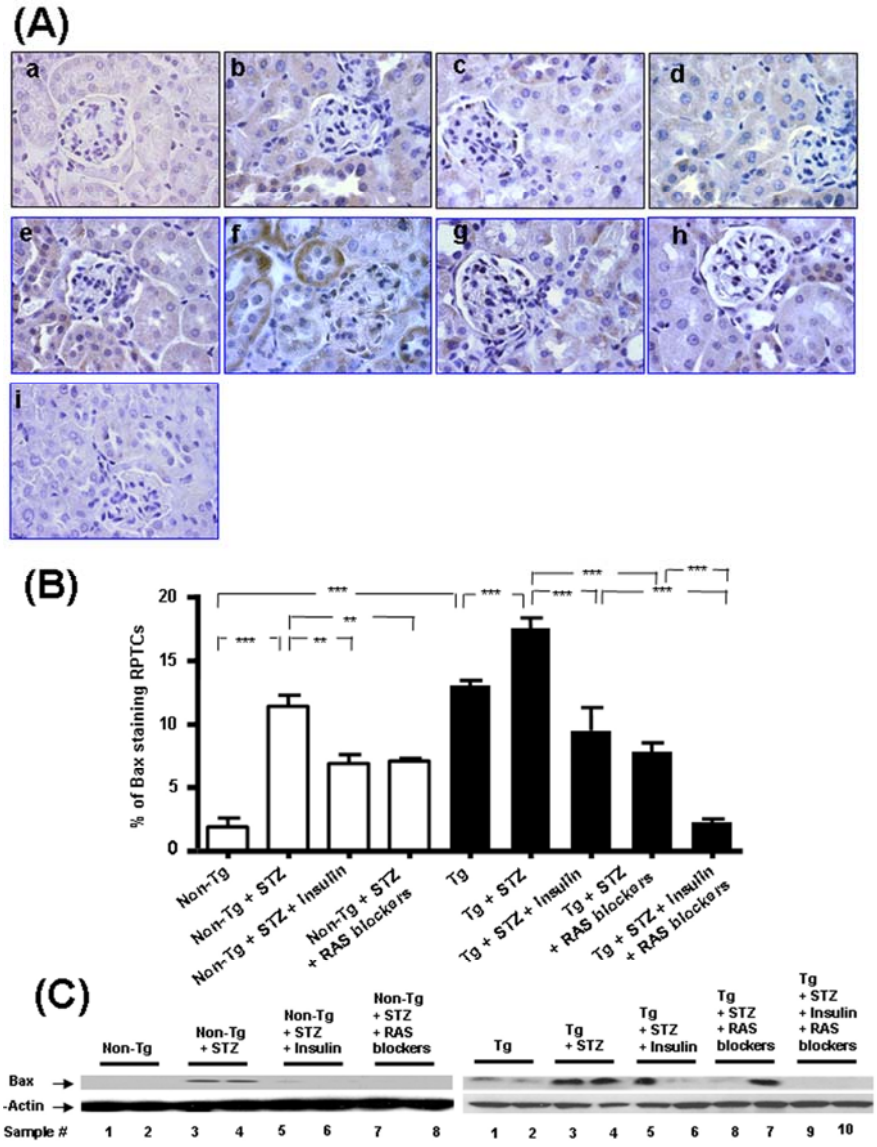

Figure 2-7. Immunohistochemical staining and Western Blots of Bax in male non-Tg and Tg mouse kidneys after 4 weeks of STZ-induced diabetes with or without treatment with insulin or RAS blockers or insulin plus RAS blockers employing rabbit anti-Bax antibodies. A. a, non-diabetic control mouse; b, STZ-induced diabetes ; c, insulin-treated, diabetic mouse; d, RAS blockers-treated, diabetic mouse; e, non-diabetic Tg mouse; f, STZ-induced diabetic Tg mouse; g, insulin-treated, STZ-induced diabetic mouse; h, RAS blockers-treated, diabetic Tg mouse; i, insulin plus RAS blockers-treated, diabetic Tg mice. Antibody dilution 1:50 and magnification x 200. B. Semi-quantitative analysis of Bax staining RPTCs from male non-Tg and Tg mouse kidneys after 4 weeks of STZ-induced diabetes with or without treatment with insulin or RAS blockers or insulin plus RAS blockers. All data are expressed as means ± SD, N=8 (*$p<0.05$, **$p<0.01$; ***$p<0.005$). Non-Tg (empty bars) and Tg (solid bars) mice. C. Western blot analysis of Bax expression (antibody dilution 1:1000) in mouse RPT extracts of male non-Tg and Tg mice.

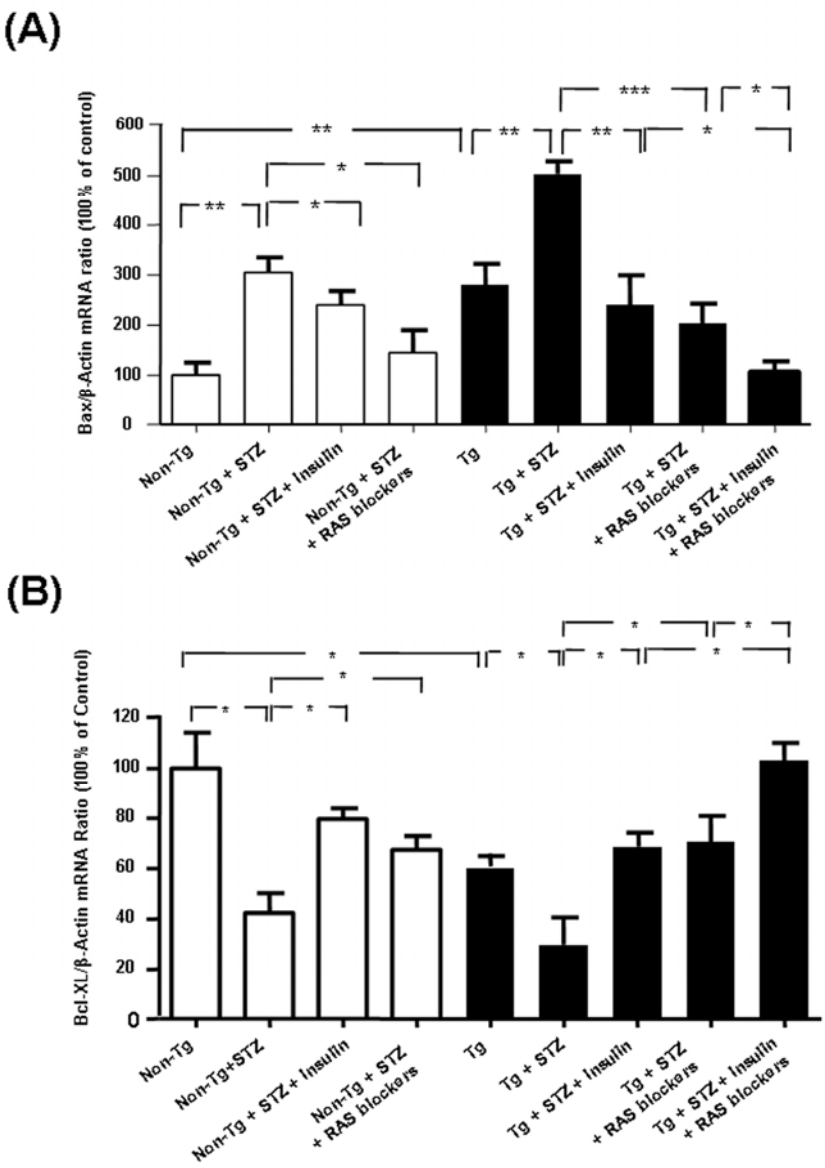

Figure 2-8. RT-qPCR assays of Bax and Bcl-xL mRNA expression in RPTs of non-Tg and Tg mice after 4 weeks of STZ-induced diabetes with or without treatment with insulin or RAS blockers or insulin plus RAS blockers. Bax and β-actin or Bcl-xL and β-actin mRNAs were run simultaneously in the same RT-qPCR assay. The CT (threshold cycle) value was measured to determine the starting copy number of target genes using the standard curve. Lower CT values indicate higher amounts of PCR products. Bax and Bcl-xL mRNA levels were normalized by corresponding β-actin mRNA levels. Bax and Bcl-xL mRNA levels in non-diabetic non-Tg were considered as 100%. All data are expressed as means ± S.D., N=8 (*$p<0.05$, **$p<0.01$; ***$p<0.005$; N.S., not significant). Non-Tg (empty bars) and Tg (solid bars) mice.

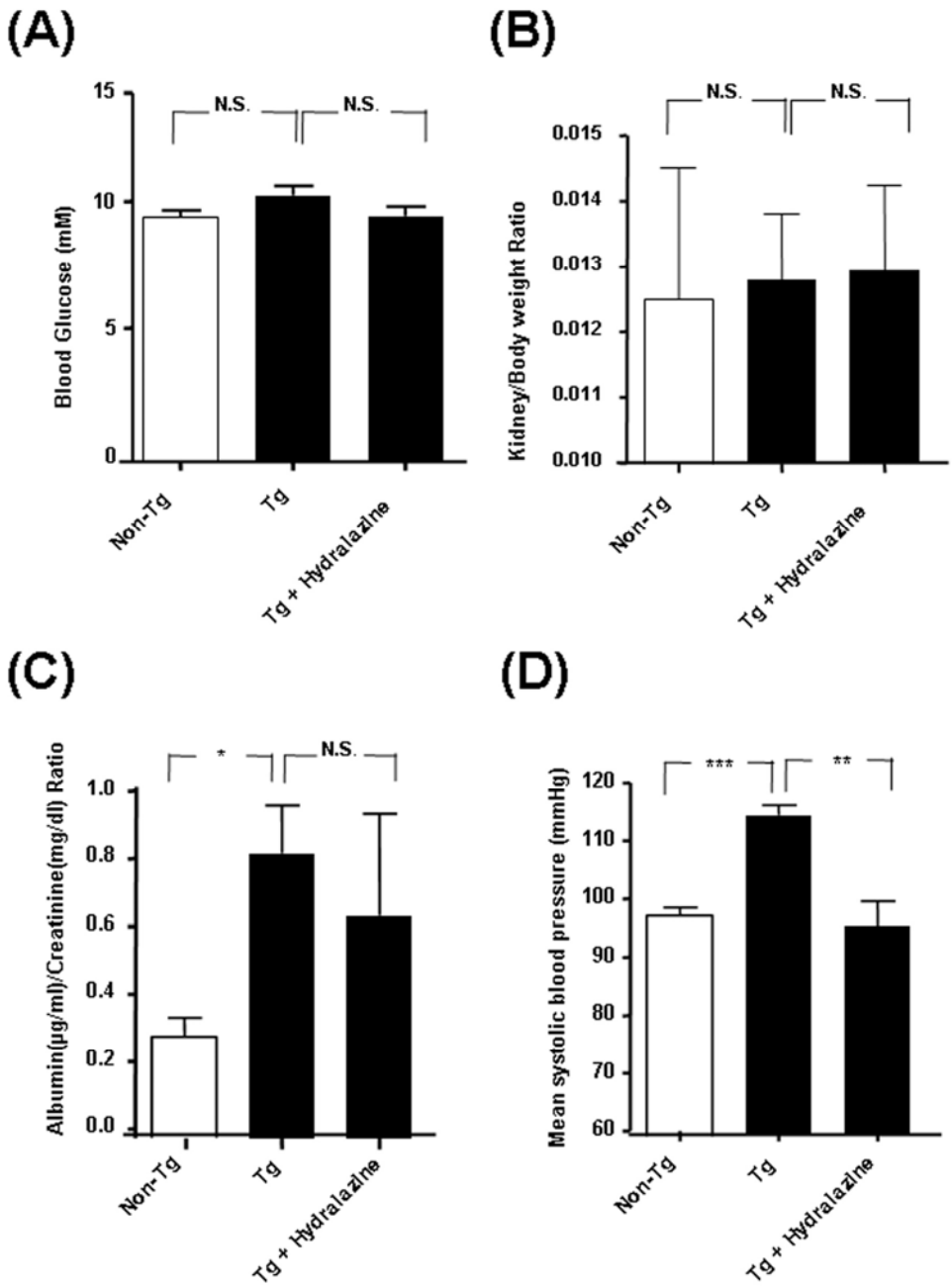

Figure 2-9. Blood glucose, kidney/body weight ratio, urinary albumin/creatinine ratio, and mean systolic blood pressure in male non-Tg and Tg mice with or without hydralazine treatment. (A) Blood glucose. Blood glucose was measured in the morning without fasting at the end of the study. (B) Kidney-to-body weight ratio. The kidney-to-body weight ratio was measured as the weight of 2 kidneys per body weight. (C) Urinary albumin (μg/ml)/creatinine (mg/dL) ratio. (D) Mean systolic blood pressure. All data are expressed as means ± SD, N=8 (*$p<0.05$, **$p<0.01$; ***$p<0.005$; N.S., not significant). Non-Tg (empty bars) and Tg (solid bars) mice.

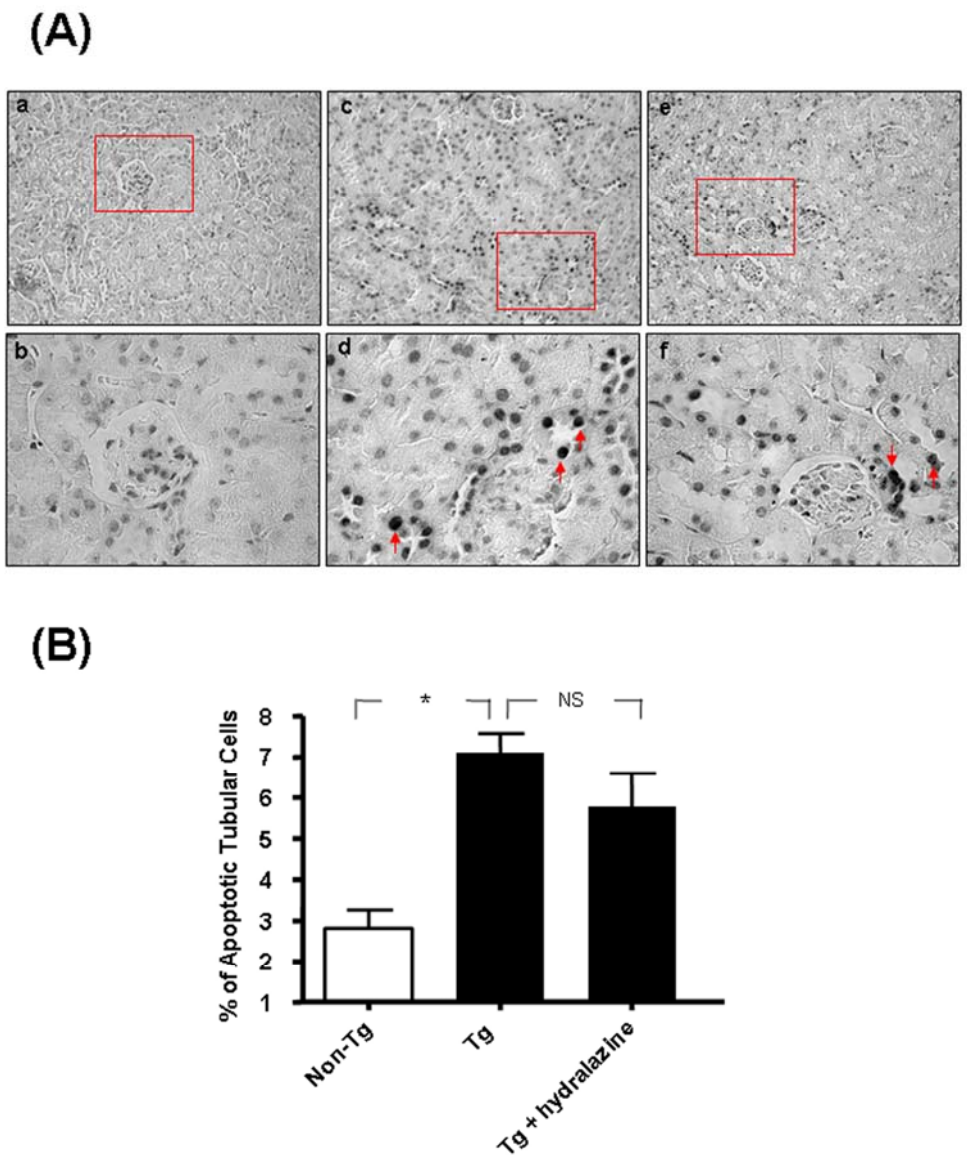

Figure 2-10. TUNEL assay of apoptotic RPTCs in male non-Tg and Tg mouse kidneys with or without hydralazine treatment. A.Non-Tg control mouse(a and b); Tg mouse(c and d); Tg mouse with hydralazine treatment(e and f). B. Semi-quantitative analysis of apoptotic RPTCs. All data are expressed as means ± SD, N=8 (*$p<0.05$, N.S., not significant). Non-Tg (empty bars) and Tg (solid bars) mice. Note: in a,c and e, magnification X200; in b,d and f, magnification X600.

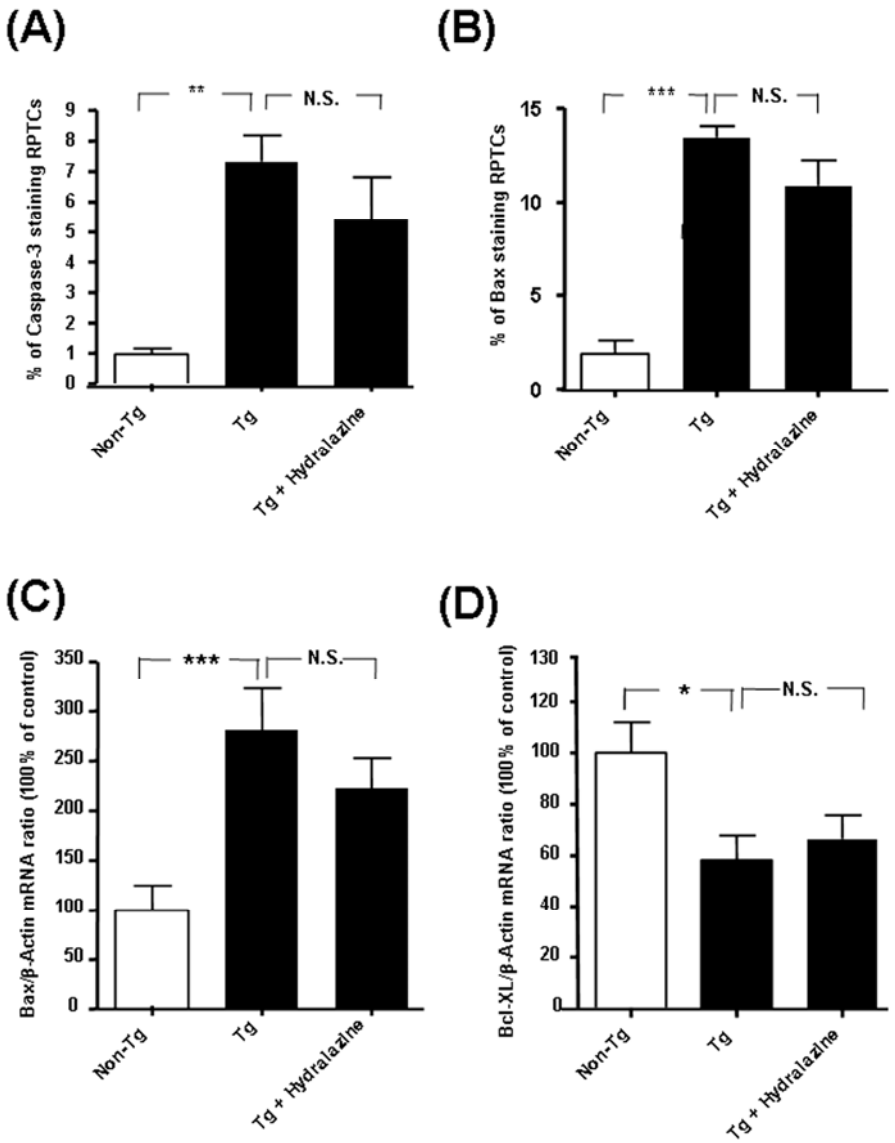

Figure 2-11. Bar graph showing semi-quantitative analysis of active caspase-3 and Bax staining in RPTCs and RT-qPCR assay of Bax and Bcl-XL mRNA expression in RPTs of male non-Tg and Tg mice with or without hydralazine treatment. (A) Active caspase-3 staining RPTCs. (B) Bax staining RPTCs. (C) Bax mRNA expression. (D) Bcl-XL mRNA expression. Urinary albumin (μg/ml)/creatinine (mg/dL) ratio. (D) Mean systolic blood pressure. The relative densities of Bax (C) and Bcl-xL (D) mRNA were normalized with control β-actin mRNA. Bax and Bcl-xL mRNA levels in non-diabetic non-Tg were considered as 100%. All data are expressed as means ± S.D., N=8 (*$p<0.05$, **$p<0.01$; ***$p<0.005$; N.S., not significant). Non-Tg (empty bars) and Tg (solid bars) mice.

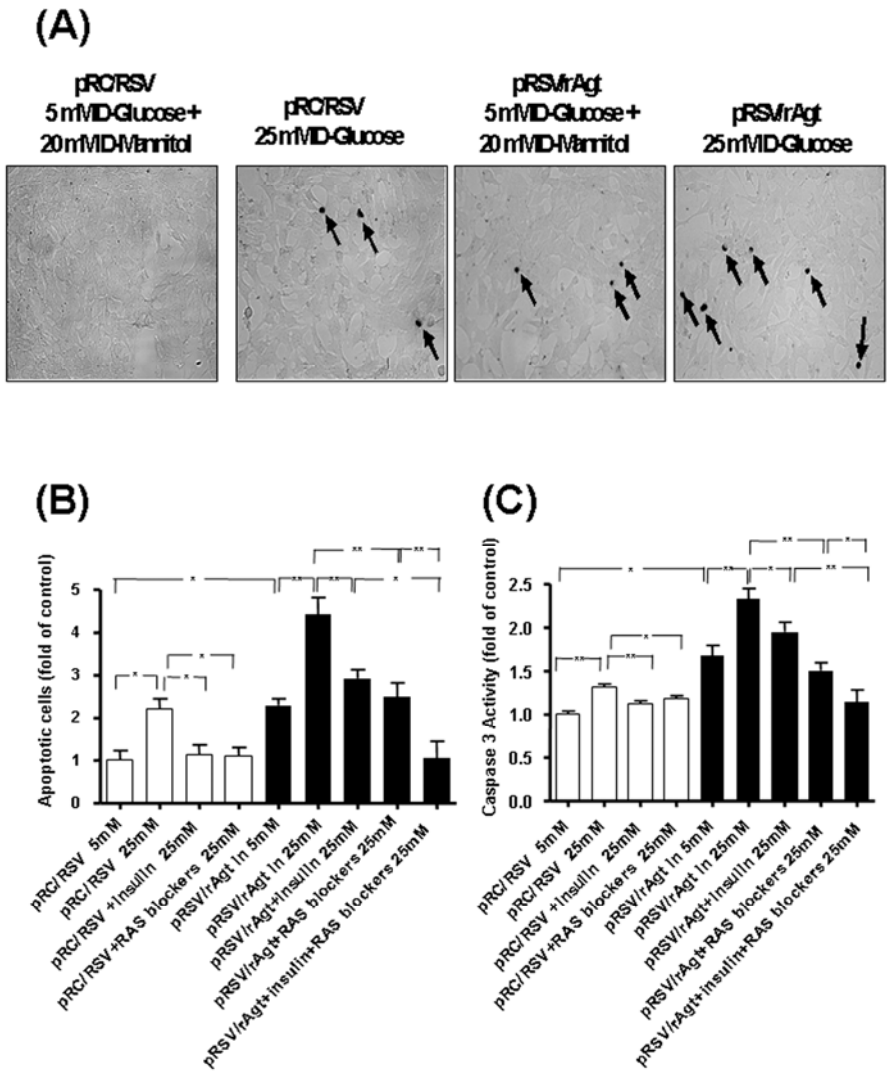

Figure 2-12. Effect of high glucose on RPTC apoptosis and caspase-3 activity in stable transfectants with or without treatment with insulin or RAS blockers or insulin plus RAS blockers. pRC/RSV and pRSV/rAgt stable transfectants were plated at a density of 1 x 10^4 cells in 6-well plates in DMEM supplemented with 5% FBS. When the cells reached 60% confluence, the medium was changed to serum-free DMEM and incubated overnight. Then, the cells were incubated for 24 h in normal glucose (5 mM D-glucose plus 20 mM D-mannitol) or high glucose (25 mM D-glucose) medium plus 0.1% depleted FBS in the absence or presence of insulin (10^{-6} M) or RAS blockers (losartan, 10^{-6} M and perindopril, 10^{-4} M) or insulin plus RAS blockers, and analyzed for apoptosis or caspase-3 activity. A. TUNEL assay of pRC/RSV and pRSV/rAgt stable transfectants in normal or high glucose medium. B. Semi-quantitative analysis of apoptotic RPTCs. C. Caspase-3 activity. The graphs represent the mean ± S.D. of 4 independent experiments and each treatment group was assayed in duplicate (*$p<0.05$, **$p<0.01$). pRC/RSV stable transfectants (empty bars) and pRSV/rAgt stable transfectants (solid bars).

Chapter 3: Article 2

Apocynin attenuates tubular apoptosis and tubulointerstitial fibrosis in transgenic mice independent of hypertension

Apocynin attenuates tubular apoptosis and tubulointerstitial fibrosis in transgenic mice independent of hypertension

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3.1 ABSTRACT

Angiotensin II stimulates the formation of reactive oxygen species by increased NADPH oxidase activity which contributes to pro-apoptotic and pro-fibrotic mechanisms critical in renal injury. Here we determine if apocynin, an inhibitor of NADPH oxidase, interferes with the action of the intrarenal renin-angiotensin system to minimize the progression of renal disease. Transgenic mice that overexpress rat angiotensinogen in their proximal tubule cells were given either apocynin, perindopril, hydralazine while untreated or apocynin-treated non-transgenic littermates served as additional controls. Untreated transgenic mice had significant elevations of their systolic blood pressure, albuminuria, reactive oxygen species production, NADPH oxidase activity, tubular apoptosis, active caspase-3, Bax, transforming growth factor-β1, plasminogen activator inhibitor-1, extracellular matrix proteins, collagen type IV and phosphorylated p47phox expression compared to untreated non-transgenic mice. Apocynin and perindopril blunted these changes, however, apocynin had no effect on the systolic blood pressure while hydralazine prevented hypertension and tubulointerstitial fibrosis but not proximal tubule cell apoptosis. Our study shows that the intrarenal renin-angiotensin system stimulates proximal tubule cell apoptosis and tubulointerstitial fibrosis, in part, by enhanced NADPH oxidase activity and reactive oxygen species generation independent of systemic hypertension.

3.2 INTRODUCTION

While the progression of chronic renal failure may be initiated by glomerular injury, studies over the past three decades have demonstrated that tubulointerstitial injury, characterized by interstitial fibrosis and tubular atrophy, may be a better predictor of renal disease progression than glomerular pathology (1-6). Tubular atrophy has been observed in various experimental models of nephropathy as well as in human renal diseases (7-12), including diabetic nephropathy (13, 14). However, the pathogenesis of tubular atrophy remains poorly understood.

Apoptosis, postulated to be involved in the pathogenesis of tubular atrophy, mediates renal cell death in various renal diseases, including glomerulosclerosis (15), polycystic kidney disease (16), ischemic renal disease (17), allograft nephropathy (18), and diabetes (19). The concept that apoptosis may mediate tubular atrophy is appealing because, unlike ischemia, which commonly leads to necrosis, apoptosis promotes cell removal with minimal inflammation (20). The prevalence of apoptotic renal proximal tubular cells (RPTCs) without concomitant RPTC hyperplasia suggests that net RPTC deletion by apoptosis may be important in the pathogenesis of tubular atrophy in chronic kidney diseases.

Enhanced reactive oxygen species (ROS) production also has been implicated in the progression of various renal diseases (21-23), including diabetic nephropathy (24). *In vitro,* ROS contribute to apoptosis of podocytes, mesangial and tubular cells exposed to high glucose (25-27). Angiotensin II (Ang II) is a potent stimulator of ROS generation via heightened NADPH oxidase activity in mesangial cells and RPTCs, and antioxidants appear to provide renal protection, in part, by ameliorating oxidative stress induced by Ang II (28-30). Such observations strongly indicate a causal link between Ang II, ROS and apoptosis within the kidney.

We previously documented that transgenic (Tg) mice overexpressing rat angiotensinogen (Agt), the sole precursor of angiotensins, in their RPTCs are prone to develop hypertension, albuminuria and renal injury (31). The present study investigated whether ROS generation mediates the pro-apoptotic and pro-fibrotic effects engendered by upregulation of the intrarenal renin-angiotensin system (RAS) in proximal tubules *in vivo,* and whether inhibition of NADPH oxidase with apocynin (an inhibitor of NAPDH oxidase) could prevent RPTC apoptosis, independently of systemic hypertension.

3.3 RESULTS

ROS Generation, Albuminuria and Renal Size in Tg Mice

Tg mice exhibited significantly augmented in ROS generation in their RPTCs (Figure 1A) and increased albumin/creatinine ratios as compared to non-Tgs (Figure 1B), though their kidney/body weight ratios were similar (Figure 1C). Treatment with either apocynin or perindopril, but not with hydralazine, normalized ROS generation and the urinary albumin/creatinine ratio, but had no effect on the kidney/body weight ratio (Figures 1A, 1B and 1C).

Hypertension in Tg Mice

Mean systolic blood pressure (SBP) in male Tg mice increased from week 11 of life, becoming statistically significant at week 12 and thereafter (Figure 2A). Perindopril and hydralazine (commenced at week 13) normalized the SBP of Tg mice after 1 week of treatment (Figure 2A). In contrast, apocynin did not significantly reduce SBP in Tg mice (Figures 2A and 2B).

Renal Morphology

Renal damage was evident in Tg mice (Figure 3A, b) as compared to non-Tg mice (Figure 3A, a). Histologic findings included vacuoles and loss of the brush border in RPTCs, a large number of detached cells and accumulation of cellular debris in the tubular lumen in Tg kidneys, indicating RPTC damage. Some RPTCs were flattened or atrophied. Treatment with apocynin (Figure 3A, c) or perindopril (Figure 3A, d) markedly attenuated these abnormalities in Tg mice, whereas hydralazine (Figure 3A, e) had minimal effect.

RPTC volume was significantly higher in untreated Tg mice than in non-Tg littermates (Figure 3B). Treatment with apocynin, perindopril or hydralazine effectively attenuated this change. Glomerular volume did not differ significantly between Tg and non-Tg mice, and was unaffected by apocynin, perindopril or hydralazine treatment (Figure 3C).

Tubular Apoptosis

Apoptotic RPTCs were more abundant in Tg (Figure 4A, b) than non-Tg mice (Figure 4A, a). Apocynin (Figure 4A, c) and perindopril (Figure 4A, d)

effectively attenuated RPTC apoptosis, while hydralazine was without effect (Figure 4A, e). Semi-quantitative estimation of the number of apoptotic RPTCs confirmed these findings (Figure 4B). Apoptotic RPTCs were detected in at least 16% and (6%) of tubules at the glomerulo-tubular (G-T) junction in Tg and non-Tg kidneys, respectively (Figure 4C). Treatment with apocynin or perindopril significantly reduced the number of apoptotic RPTCs at the G-T junction to 3% and 5%, respectively, while hydralazine had little effect (14%).

Immunohistochemistry revealed increased staining for active caspase-3 (Figure 5A, b) and Bax (Figure 5B, b) in RPTCs of Tg mice compared to non-Tg mice (Figure 5A, a and Figure 5B, a). Treatment with apocynin (Figure 5A, c and Figure 5B, c) or perindopril (Figure 5A, d and Figure 5B, d) effectively attenuated these changes, whereas hydralazine had no effect (Figure 5A, e and Figure 5B, e). Similar trends were observed in caspase-3 activity assays (Figure 5C) and Western blotting of Bax (Figure 5D) from isolated renal proximal tubules (RPTs). Taken together, these data demonstrate that apocynin and the RAS blocker perindopril effectively prevented RAS-induced RPTC apoptosis, while hydralazine had minimal effect.

Tubulointerstitial Fibrosis

Tg kidneys exhibited enhanced expression of extracellular matrix proteins (Figure 6A, b), collagenous components (Figure 6B, b) and immunoreactive collagen type IV (Figure 6C, b) as compared to the kidneys of non-Tg mice (Figure 6A, a, Figure 6B, a and Figure 6C, a). These changes were attenuated by apocynin (Figure 6A, c, Figure 6B, c and Figure 6C, c), perindopril (Figure 6A, d, Figure 6B, d and Figure 6C, d) and hydralazine (Figure 6A, e, Figure 6B, e and Figure 6C, e) as quantitated in sections stained with Masson's trichrome (Figure 6D) or immunoreactive collagen type IV (Figure 6E).

Similarly, Tg kidneys also showed higher expression of transformining growth factor-beta 1 (TGF-β1) and plasminogen activator inhibitor-1 (PAI-1) (Figures 7A, b and 7B, b) than non-Tg kidneys (Figures 7A, a and 7B, a). Treatment with apocynin (Figures 7A, c and 7B, c), perindopril (Figures 7A, d

and 7B, d) or hydralazine (Figures 7A, e and 7B, e) attenuated these changes. TGF-β1 and PAI-1 expression was quantitated by Western blotting (Figures 7C and Figure 7D).

Effect of Apocynin and Perindopril on Bax, Bcl-xL, TGF-β1 and PAI-1 mRNA Expression in Tg Mice

Bax mRNA expression was significantly higher (Figure 8A), whereas Bcl-xL mRNA expression was significantly lower (Figure 8B) in Tg than in non-Tg RPTs. Treatment with apocynin or perinodpril markedly reversed these changes, whereas hydralazine had no detectable effects. In contrast, all agents attenuated the increases in TGF-β1 mRNA (Figure 8C) and PAI-1 mRNA (Figure 8D) expression in Tg RPTs.

Effect of Apocynin on NADPH Oxidase Activity and p47phox Expression in Tg Mice

ROS generation and NADPH oxidase activity were significantly higher in Tg than in non-Tg RPTs (Figures 9A and 9B). Treatment with apocynin effectively inhibited ROS generation and NADPH activity in Tg RPTs, but not in non-Tg RPTs. Furthermore, phosphorylated (p)-p47phox and p47phox expression in membrane (Figure 9C) and cytosolic (Figure 9D) fractions of Tg RPTs was significantly higher in Tg RPTs than in non-Tg RPTs. Apocynin effectively attenuated p-p47phox and p47phox expression in membrane fractions of Tg RPTs without affecting non-Tg RPTs (Figure 9C). In contrast, apocynin did not effect p-p47phox and p47phox expression in cytosolic fractions of Tg and non-Tg RPTs (Figure 9D).

3.4 DISCUSSION

The present study demonstrates that apocynin treatment prevents RPTC apoptosis in Agt-Tg mice independently of systemic hypertension. These findings indicate that ROS generation via intrarenal RAS activation likely plays a centrol role in the induction of RPTC apoptosis.

Our Tg model specifically expresses Agt in RPTCs but not in other tissues (31). Our data significantly demonstrate elevated ROS generation in RPTs of these Tg mice compared to non-Tg mice, consistent with previous reports on Ang II induction of ROS generation in rat (32) and human RPTCs (33). The observation that apocynin and perindopril blocked ROS generation in RPTs of Tg mice, whereas hydralazine had no effect, indicates that enhanced ROS generation and albuminuria in Tg mice may be attributed to augmented intrarenal RAS activation *per se* rather than to increased SBP. Unlike hydralazine, both apocynin and perindopril reversed albuminuria in Tg mice.

Baseline SBP was significantly higher in Tg than in non-Tg mice. Between weeks 12 and 20, the SBP in Tg mice rose on average by 20 mm Hg ($p<0.05$) as compared to non-Tg mice (mean SBP was 100 and 120 mm Hg in non-Tg and Tg mice, respectively). Hydralazine and perindopril effectively attenuated SBP after 1 week of treatment. Surprisingly, apocynin did not lower SBP in Agt Tg mice. Previous studies have reported that apocynin decreased blood pressure in Dahl salt-sensitive hypertensive rats (34), aldosterone-induced hypertensive rats (35) and Ang II-induced hypertensive rats (36). The reasons for this apparent discrepancy are unknown. One possible explanation is that Ang II derived from Agt in RPTCs (i.e., Ang II generated via RPTC renin and angiotensin-converting enzyme (ACE) in RPTCs (37)) might affect SBP through direct vasoconstriction of glomerular arterioles via the Ang II AT_1-receptor independently of ROS generation. This possibility is further supported by the observation that hydralazine prevented hypertension in Tg mice without ameliorating intrarenal abnormalities. Another possibility is that apocynin is not effective in blocking the membrane translocation of p-p47phox in glomerular arteriole smooth muscle cells, although it does block the translocation of p-p47phox in RPTs, as seen in our study. Indeed, Heumuller et al. (38) recently reported that apocynin is not an effective inhibitor of vascular NADPH oxidase due to the absence of myeloperoxidase in vascular smooth muscle cells to convert apocynin into active apocynin dimer. In contrast, myeloperoxidase

protein has been detected in RPTs of patients with membranous glomerulopathy (39), consistent with formation of active apocynin.

While oxidative stress is associated with hypertension, it remains unclear whether ROS could initiate the development of hypertension. In clinical studies, treatment with antioxidants failed to decrease high blood pressure (40, 41). Likewise, apocynin did not affect hypertension in the present study but yet attenuated renal ROS generation. Thus, it is conceivable that oxidative stress is not the cause, but rather a consequence of hypertension, such that blocking ROS ameliorates the renal lesions from a downstream point.

Our data highlight the importance of intrarenal Agt gene expression and ROS generation in mediating RPTC hypertrophy and tubular apoptosis in the mouse kidney. Although the relationship between RPTC hypertrophy and apoptosis is not well understood, it is speculated that low cellular ROS levels induce cell hypertrophy whereas high ROS levels evoke cell apoptosis. Indeed, Griendling's group and others have shown that ROS stimulates both cellular hypertrophy and apoptosis, depending on cellular ROS levels and their molecular species (42, 43).

The numbers of terminal transferase-mediated deoxyuridine triphosphate nick-end-labeling (TUNEL)-positive RPTCs were significantly higher in Tg than in non-Tg mice. These data are consistent with previous reports concerning Ang II induction of RPTC apoptosis both in vitro and in vivo (33, 44). Consistent with the TUNEL assay, RPTs of Tg mice exhibited enhanced active caspase-3 and Bax expression. These changes were attenuated by apocynin and perindopril but not by hydralazine. Heighthened Bax expression occurred with concomitant downregulation of the Bcl-xL gene. Apocynin and perindopril but not hydralazine reversed the Bax/Bcl-xL ratio. An increased Bax/Bcl-xL ratio is consistent with promotion of apoptosis, and is a likely mechanism by which intrarenal RAS activation and ROS enhance tubular apoptosis in Tg mice.

The precise mechanism(s) by which apocynin prevents Ang II-induced ROS generation and subsequent tubular injury (albuminuria, interstitial fibrosis and RPTC apoptosis) in Tg mice is far from being fully understood. It has been

postulated that apocynin blocks NADPH oxidase assembly by interfering with p-p47phox binding to gp91 phox (45), as Ang II activates protein kinase C and subsequently phosphorylates p47phox (46). Indeed, our data support this notion. Likewise, the mechanism(s) by which ROS induce RPTC apoptosis remains undefined. One possibility is that ROS, through activating p38 mitogen-activated protein kinase signalling, stimulate p53 phosphorylation (47). Phosphorylated p53 then translocates to the nucleus and enhances Bax gene transcription (48, 49). Bax translocates to the mitochondria and binds the anti-apoptotic proteins Bcl-2 and Bcl-xL, inhibiting their protective actions on gate-keeping and, ultimately, leading to mitochondrial dysfunction and caspase-3 activation. Our data on increased Bax and caspase-3 expression, combined with decreased Bcl-xL expression in RPTs of diabetic Tg mice, lend support to this notion. Furthermore, our data indicate that enhanced intrarenal RAS activation stimulates Bax mRNA and protein expression in Tg mice.

The present results may have clinical implications. Since tubular apoptosis is detectable in various renal diseases (7-14) and tubular atrophy appears to be a better indicator of disease progression than glomerular pathology (4, 19, 50, 51), we postulate that RPTC apoptosis may be an initial step leading to tubular atrophy and that ROS is one of the key mediators of this process.

In summary, our study indicates an important role for ROS in albuminuria, interstitial fibrosis and RPTC apoptosis in Agt-Tg mice independently of systemic hypertension *in vivo*. Our Tg mice may be a useful animal model to study the mechanism (s) of Ang II action on ROS generation in the kidney. Our data suggest that selective inactivation of renal NADPH oxidase may provide a novel therapeutic target for attenuating or reversing nephropathy, including tubular atrophy, in various renal diseases.

3.5 MATERIALS AND METHODS

D (+)-glucose, bovine serum albumin (fraction V), apocynin (4-hydroxy-3-methoxyacetophenone, an orally-active and selective inhibitor of NADPH oxidase), hydralazine (a vasodilator), and monoclonal antibodies against β-actin

were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Perindopril (an ACE inhibitor) was obtained from Servier Amérique, Laval, QC, Canada. Anti-cleaved caspase-3, anti-Bax polyclonal antibody and monoclonal anti-collagen type IV antibody were procured from New England Biolabs Ltd. (Pickering, ON, Canada), BD Pharmingen (San Diego, CA, USA) and Chemicon International, Inc. (Temecula, CA, USA), respectively. Protein G plus beads, anti-TGF-β1, anti-PAI and anti-p47phox polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-phosphoserine monoclonal antibody was obtained from BIOMOL International, Inc. (Plymouth Meeting, PA, USA). Normal glucose (5 mM) Dulbecco's modified Eagle's Medium (DMEM, Catalogue No. 12320) and 100 × penicillin/streptomycin were bought from InVitrogen, Inc. (Burlington, ON, Canada).

Agt-Tg Mice

Tg mice (C57Bl6 background, line #388) were created in our laboratory (J.S.D.C.) by inserting rAgt cDNA fused with a HA tag (a sequence encoding amino acid residues 98-106 (YPYDVPDYA) of human influenza virus hemagglutinin) at the 3' terminal into a construct containing the kidney-specific androgen-regulated protein promoter specific to RPTCs (31). Non-Tg, sex-matched littermates served as controls. All animals received standard mouse chow and water *ad libitum*. The experimental procedures were approved by the Animal Care Committee of the CHUM.

Physiological Studies

Male adult Tg mice (age 8-10 weeks) were divided into 4 groups (8 mice per group): 1. Tg mice treated with sterile water sweetened with NutraSweet; 2. Tg mice treated with apocynin (Sigma-Adrich Canada Ltd.) at 2.4 g/l dissolved in sterile water (sweetened with NutraSweet), as established by Susztak et al. (27); 3. Tg mice given perindopril (4 mg/kg/day) in drinking water; 4. Tg mice given

hydralazine (15 mg/kg/day) in drinking water. Non-Tg littermates receiving no drug or treated with apocynin served as additional controls.

Three days after treatment, SBP was measured with a BP-2000 tail-cuff pressure device (Visitech Systems, Apex, NC, USA) (31) in the morning, at least 2 to 3 times a week for 10 weeks. The mice were trained to this procedure for at least 15-20 min per day for 5 days prior to the first SBP reading. SBP values represent the means ± standard deviation (M ± S.D.) of 2 to 3 determinations per week per group of animals.

All animals were euthanized at age 18-20 weeks. Twenty-four h prior to euthanasia, they were housed individually in metabolic cages. Body weight was recorded. Urine was collected and assayed for albumin and creatinine (ELISA, Albuwell and Creatinine Companion, Exocell, Inc., Philadelphia, PA, USA) (31). Both kidneys were removed, decapsulated and weighed together. The left kidneys were processed for histology and apoptosis study, and the right kidneys were used for isolation of RPTs by Percoll gradient (31).

Histological Studies

Kidneys were collected in Tissue-Tek cassettes (VWR Canlab, Montreal, QC, Canada), dipped immediately in ice-cold formaldehyde (10% in phosphate-buffered saline (PBS)) and fixed for 24 h at 4^{o}C. The cassettes were then processed by the CHUM Pathology Department. Tissue blocks were cut with a microtome to obtain the desired tissue specimens. Tissue sections (4-5 sections per kidney) from 8 animals per group were stained with hematoxylin/eosin or periodic acid Schiff (PAS) or Masson's trichrome. Masson's trichrome-stained images were quantified by NIH ImageJ software (http://rsb.info.nih.gov/ij/). Briefly, 10-15 fields per animal were randomly selected from each group. The collected images were changed into 8-bit grayscale by the software. Then, a threshold was determined to filter particles outside the color and intensity range. This threshold was applied to all images, which were analyzed by calculating the total area covered by colored particle deposition and expressed as 'arbitrary units'.

The mean volume of 30 random glomeruli per mouse was assessed by Weibel's method (52) with Motic Images Plus 2.0 image analysis software (Motic, Richmond, BC, Canada). RPTC volume was measured from 100 RPTCs of 50 RPTs per mouse, respectively, with the same software. Outer cortical RPTs with similar cross-sectional views and clear nuclear structure were selected. Mean cell volume was estimated by the Nucleator method (53).

TUNEL Assay and Immunohistochemical Staining

The percentage of apoptotic RPTCs (TUNEL kit, Roche Diagnostics, Laval, QC, Canada) was estimated semi-quantitatively (54, 55). Briefly, 10-15 fields per animal were randomly selected from each group. Then, the total number of apoptotic RPTCs was divided by the total number of RPTCs counted and multiplied by 100 to derive the % of apoptotic-positive RPTCs. Similarly, the % of G-T junctions containing apoptotic RPTCs was quantified by counting the total number of G-T junctions containing apoptotic RPTCs divided by the total number of G-T junctions counted (approximately 20 G-T junctions counted for each group) and then multiplied by 100.

Immunohistochemical staining was performed according to the standard avidin-biotin-peroxidase complex method (ABC Staining System, Santa Cruz Biotechnology, Inc.) (31, 54). Collagen IV immuno-stained images were quantified by NIH ImageJ software, as described above for Masson's trichrome staining.

ROS Generation, NADPH Oxidase Activity and Caspase-3 Activity Assay

Aliquots of freshly-isolated proximal tubules were prepared immediately for ROS measurement by the lucigenin method (54, 56). Briefly, RPTs were washed in modified Krebs buffer containing NaCl (130 mM), KCl (5 mM), $MgCl_2$ (1 mM), $CaCl_2$ (1.5 mM), K_2HPO_4 (1 mM) and Hepes (20 mM), pH 7.4, and resuspended in 900 μl of Krebs buffer supplemented with 1 mg/ml bovine serum albumin. The cell suspension was transferred to plastic tubes and ROS production was assessed in a luminometer (LB 9507; Berthold, Wildbad,

Germany). Measurement was started by an injection of 100 µl lucigenin (final concentration 5×10^{-4} M). Photon emissions were counted every 1 min for up to 20 min. Peak emissions at 10 min were recorded from each group for comparison. Modified Krebs buffer served as a control (blank). Solutions containing lucigenin without RPTs did not display any significant interference in the lucigenin assay. ROS production in RPTs was normalized with protein concentration and expressed as relative light units per µg protein.

NADPH oxidase activity was measured by a luminescence assay (35, 57). Briefly, RPTs were homogenized in lysis buffer and assayed in 50 mM phosphate buffer, pH. 7.0, containing 1 mM EGTA, 150 mM sucrose, 5 µM lucigenin and 100 µM NADPH (final volume, 0.9 ml). The reaction was started after the addition of 100 µl (~100 µg) of homogenate protein. No activity was detected in the absence of NADPH.

Caspase-3 activity was assayed on frozen (-80ºC) mRPTs with caspase-3 assay kits (BD Bioscience Pharmingen, Mississauga, ON, Canada), as described previously (54).

Cellular Fractionation

RPTs were fractionated to membrane and cytosolic fractions by the method of Medhora et al. (58). Briefly, RPTs were sonicated for 8 seconds on ice in 25 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, 1 mM EGTA and a cocktail of protease inhibitors (10 µg/ml aprotinin, 0.5 µg/ml leupeptin, 0.7 µg/ml pepstatin and 0.5 mM phenylmethylsufonyl fluoride). Following centrifugation at 18,200 x g (13,6000 rpm) for 10 min at 4ºC, pellets containing the membrane fraction were solubilized with RIPA buffer (50 mM Tris-HCl, pH. 7.4, containing 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA, and a cocktail of protease inhibitors).

Immunoprecipitation, Western Blotting and Real Time-Quantitative Polymerase Chain Reaction (RT-qPCR) Assays for Gene Expression

Immunoprecipitation of membrane and cytosolic fractions was performed as the following: Briefly, 200 µg of membranous or cytosolic fraction was incubated with 1 µg anti-p47phox antibody for 3 hrs at 4ºC with agitation. Then, 50 µl of Protein G plus beads was added to each sample and further agitated for overnight at 4ºC. Then, the mixture was centrifuged and beads were washed 3 times in lysis buffer. Finally, 20 µl of 2x loading buffer was added to the beads, boiled at 95ºC for 5 minutes, and subjected to SDS-PAGE. Western blotting for Bax, TGF-β1, PAI-1, p47phox and phosphoserine was performed as described elsewhere (31, 54). Bax, Bcl-xL, TGF-β1 and PAI-1 mRNA expression in mRPTs was quantified by RT-qPCR with forward and reverse primers corresponding to the respective genes (Table I) (54).

Statistics

Data were expressed as mean ± S.D. and analyzed by 1-way ANOVA and the Bonferroni test. $P<0.05$ values were considered significant.

3.6 ACKNOWLEDGEMENTS

This work was supported by grants from the Kidney Foundation of Canada, the Canadian Institutes of Health Research (CIHR, MOP-84363 to J.S.D.C. and MT-12573 to J.G.F.) and the National Institutes of Health of the USA (HL-48455 to J.R.I.). The authors thank Mr. Ovid M. Da Silva, Research Support Office, Research Centre, CHUM, for editing this manuscript.

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3.8 Legends and Figures

Table 3-1. Primers for RT-PCR

TGF-β1 (S) N+1031 to N+1051 (5'-CCA AAC TAA GGC TCG CCA GTC-3')
TGF-β1 (AS) N+1301 to N+1282 (5'-GGC ACT GCT TCC CGA ATG TC-3')
(NM_011577)

PAI-1 S N+972 to N+989 (5'-CTC ATC AGA CAA TGG AAG AGC-3')
PAI-1 AS N+1331 to N+1311 (5'-CAT CAC TTG CCC CAT GAA GAG-3')
(NM_008871)

Bax (S) N+179 to N+199 (5'-TCA TGA AGA CAG GGG CCT TTT-3')
Bax (AS) N+364 to N+344 (5'-CAA TCA TCC TCT GCA GCT CCA-3')
(NM_007525)

Bcl-xL (S) N+493 to N+516 (5'-TAC CGG AGA GCG TTC AGT GAT CTA-3')
Bcl-xL (AS) N+672 to N+649 (5'-CTG CAT CTC CTT GTC TAC GCT TTC-3')
(NM_009743)

β-actin (S) N+703 to N+723 (5'-CGT GCG TGA CAT CAA AGA GAA-3')
β-actin (AS) N+839 to N+819 (5'-GCT CGT TGC CAA TAG TGA TGA-3')
(NM_007393)

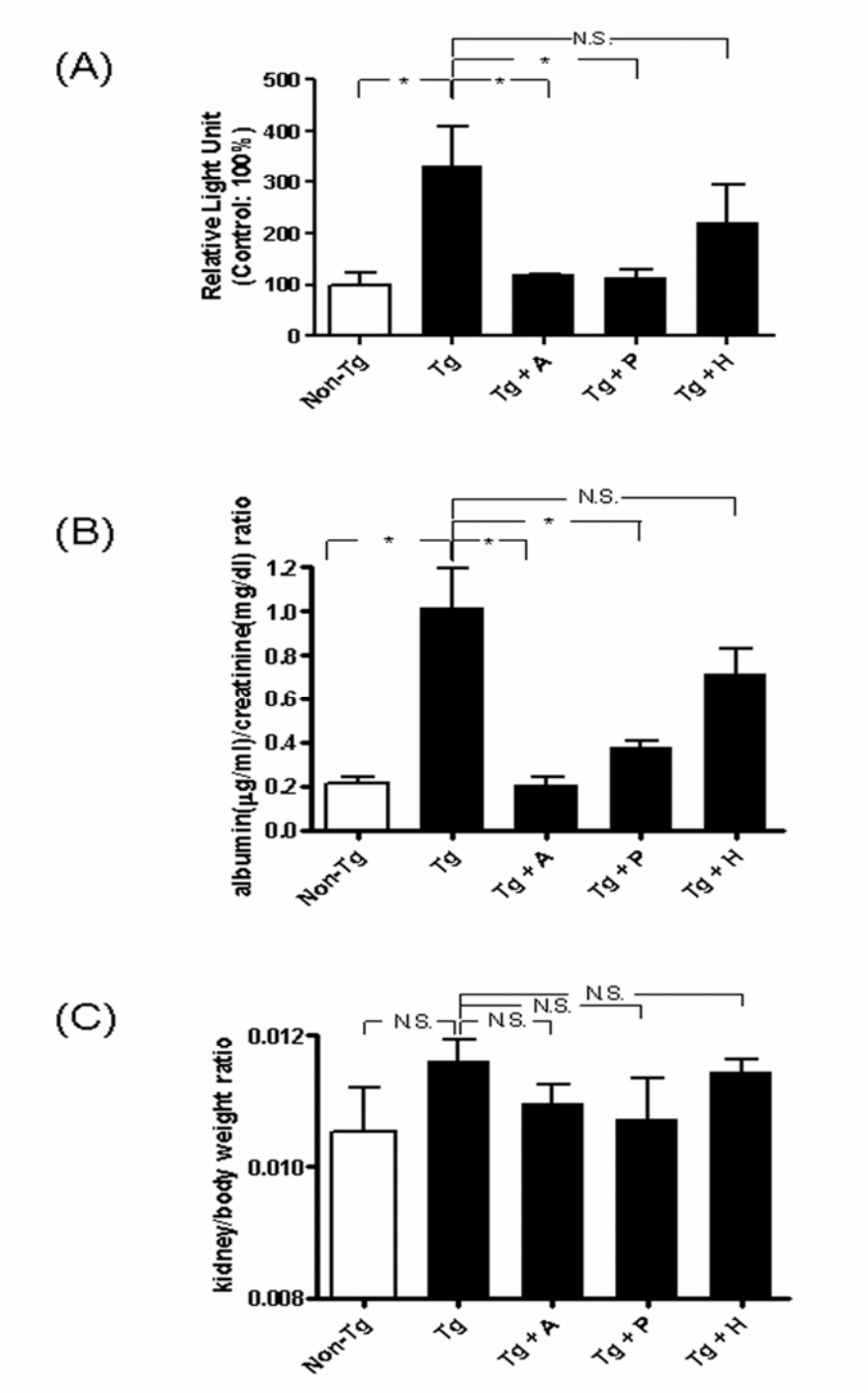


Figure 3-1. ROS generation, urinary albumin/creatinine ratio and kidney/body weight ratio in male non-Tg and Tg mice with or without apocynin, perindopril or hydralazine treatment. (A) ROS generation in RPTs; (B) Urinary albumin (μg/ml)/creatinine (mg/dl) ratio; (C) Kidney-to-body weight ratio. The kidney-to-body weight ratio was measured as the weight of 2 kidneys per body weight. All data are expressed as means ± S.D., N=8 (*$p<0.05$; N.S., not significant). Non-Tg (empty bars) and Tg (solid bars) mice. A, apocynin; P, perindopril; H, hydralazine.

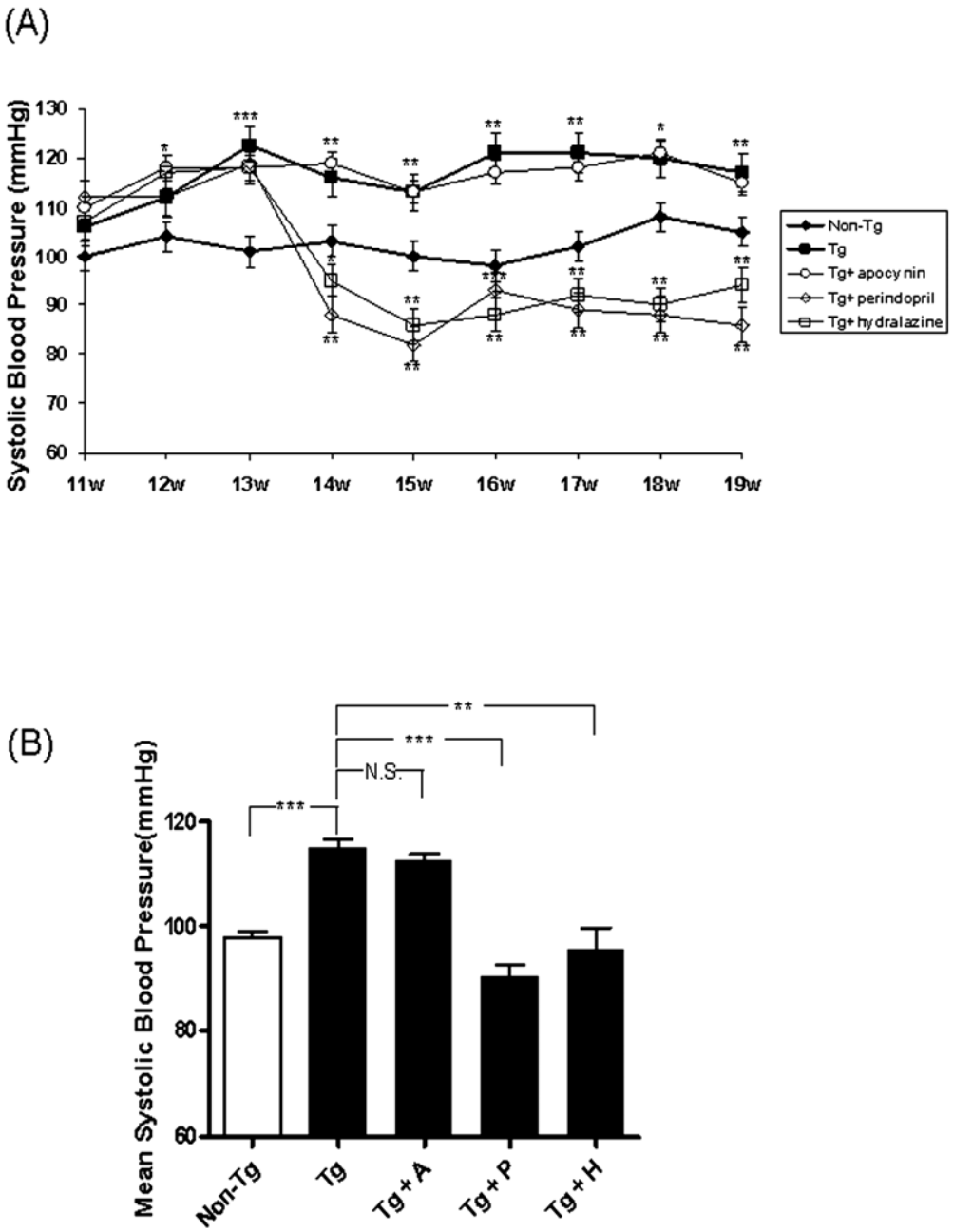


Figure 3-2. SBP in Tg and non-Tg mice. (A) Longitudinal changes in mean SBP in male non-Tg and Tg mice with or without apocynin, perindopril or hydralazine treatment for a 8-week period. Baseline SBP was measured in all mice for 5 days before the first SBP reading. Non-Tg mice (♦), Tg mice (■), and Tg mice treated with apocynin (○), perindopril (◊) or hydralazine (□) starting at week 13. (B) Cross-sectional analysis of SBP (measured 2 to 3 times per week in the morning without fasting, week 20) in non-Tg and Tg mice with or without apocynin, perindopril or hydralazine treatment. All data are expressed as means ± S.D., N=8 (*$p<0.05$; **$p<0.01$; ***$p<0.005$; N.S., not significant). Non-Tg (empty bars) and Tg (solid bars) mice. A, apocynin; P, perindopril; H, hydralazine.

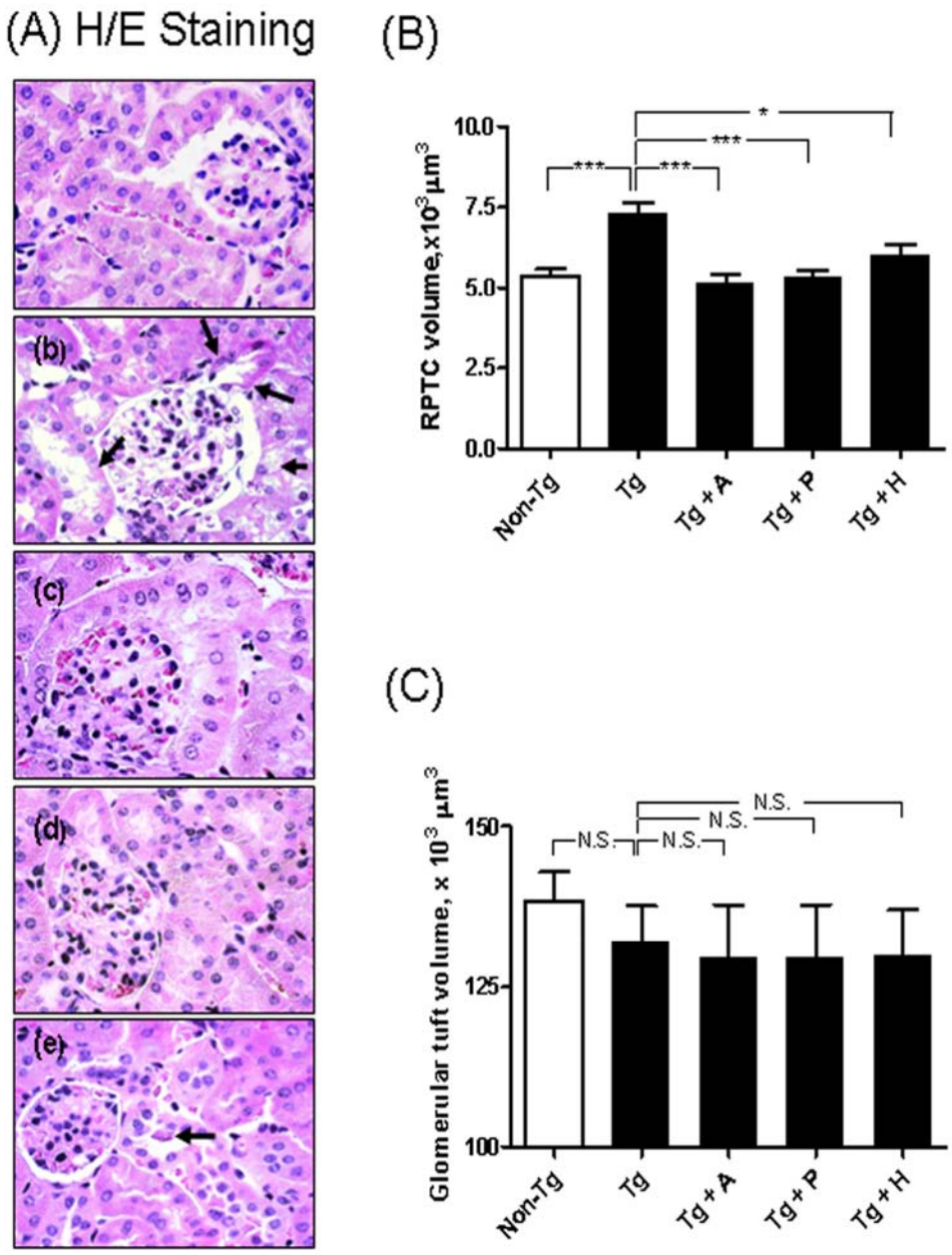


Figure 3-3. (A) Hematoxylin/eosin (H/E) staining of kidneys of male non-Tg mice and Tg mice with or without apocynin, perindopril or hydralazine treatment. A a, Non-Tg control; b, Tg mouse; c, apocynin-treated Tg mouse; d, perindopril-treated Tg mouse; e, hydralazine-treated Tg mouse. Magnification, X600. (B). Mean RPTC volume of male non-Tg and Tg mouse kidneys with or without treatment at week 20. (C). Mean glomerular volume of male non-Tg and Tg mouse kidneys with or without treatment at week 20. Arrows indicate tubular damage, i.e., RPTCs without brush border, presence of vacuoles or cellular atrophy. A, apocynin; P, perindopril; H, hydralazine.

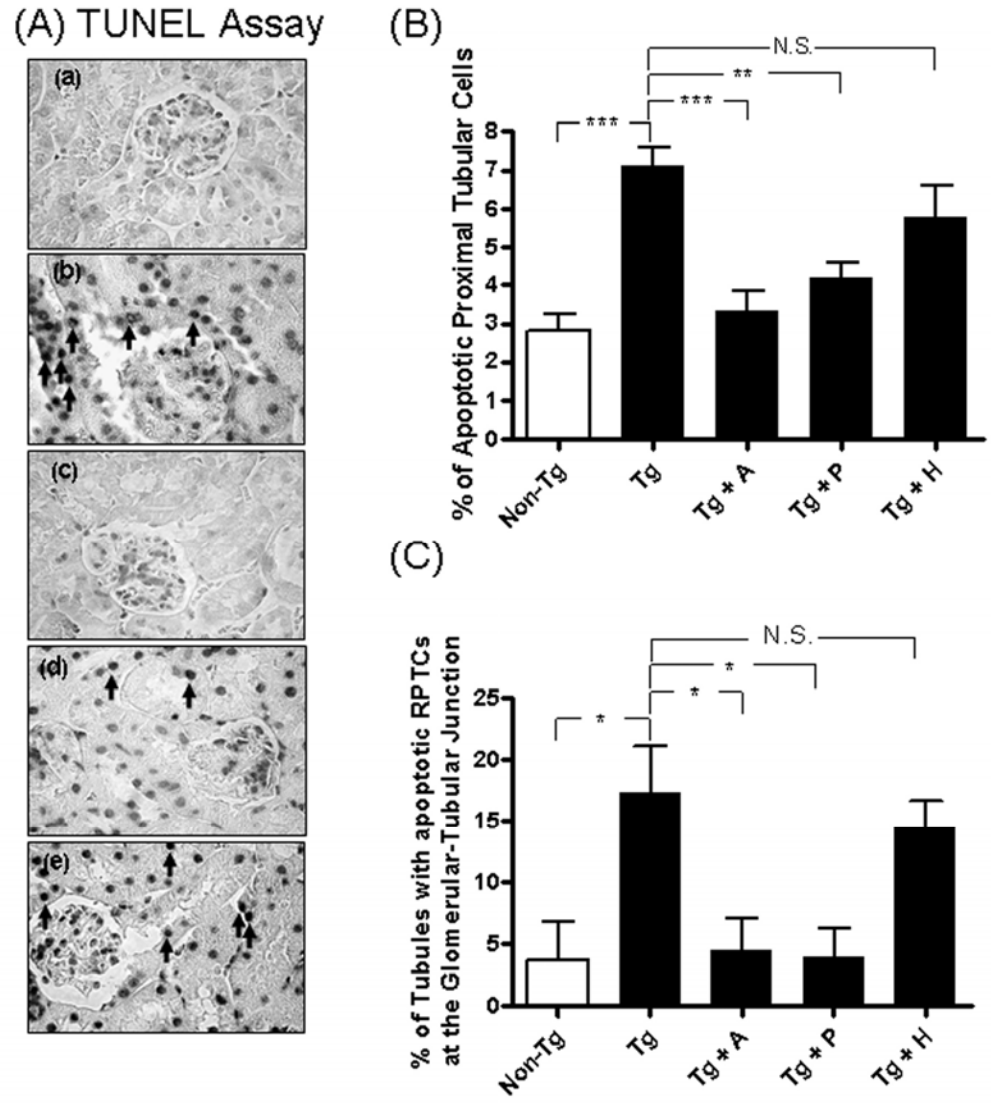


Figure 3-4. Apoptosis in male non-Tg and Tg mouse kidneys with or without apocynin, perindopril or hydralazine treatment, analyzed by TUNEL assay. (A) a, Non-Tg control; b, Tg mouse; c, apocynin-treated Tg mouse; d, perindopril-treated Tg mouse; e, hydralazine-treated Tg mouse. Magnification, x600. Arrows indicate apoptotic cells in proximal tubules. (B) Bar graph showing semi-quantitative analysis of apoptotic RPTCs from male non-Tg and Tg mouse kidneys with or without apocynin, perindopril or hydralazine treatment. (C) Bar graph showing semi-quantitative analysis of % of tubules with apoptotic RPTCs at the glomerulo-tubular (G-T) junction of 20 glomeruli from male non-Tg and Tg kidneys with or without apocynin, perindopril or hydralazine treatment. Values are expressed as means ± S.D., N=8 (*$p<0.05$; **$p<0.01$, ***$p<0.005$; N.S., not significant). Non-Tg (empty bars) and Tg (solid bars) mice. A, apocynin; P, perindopril; H, hydralazine.

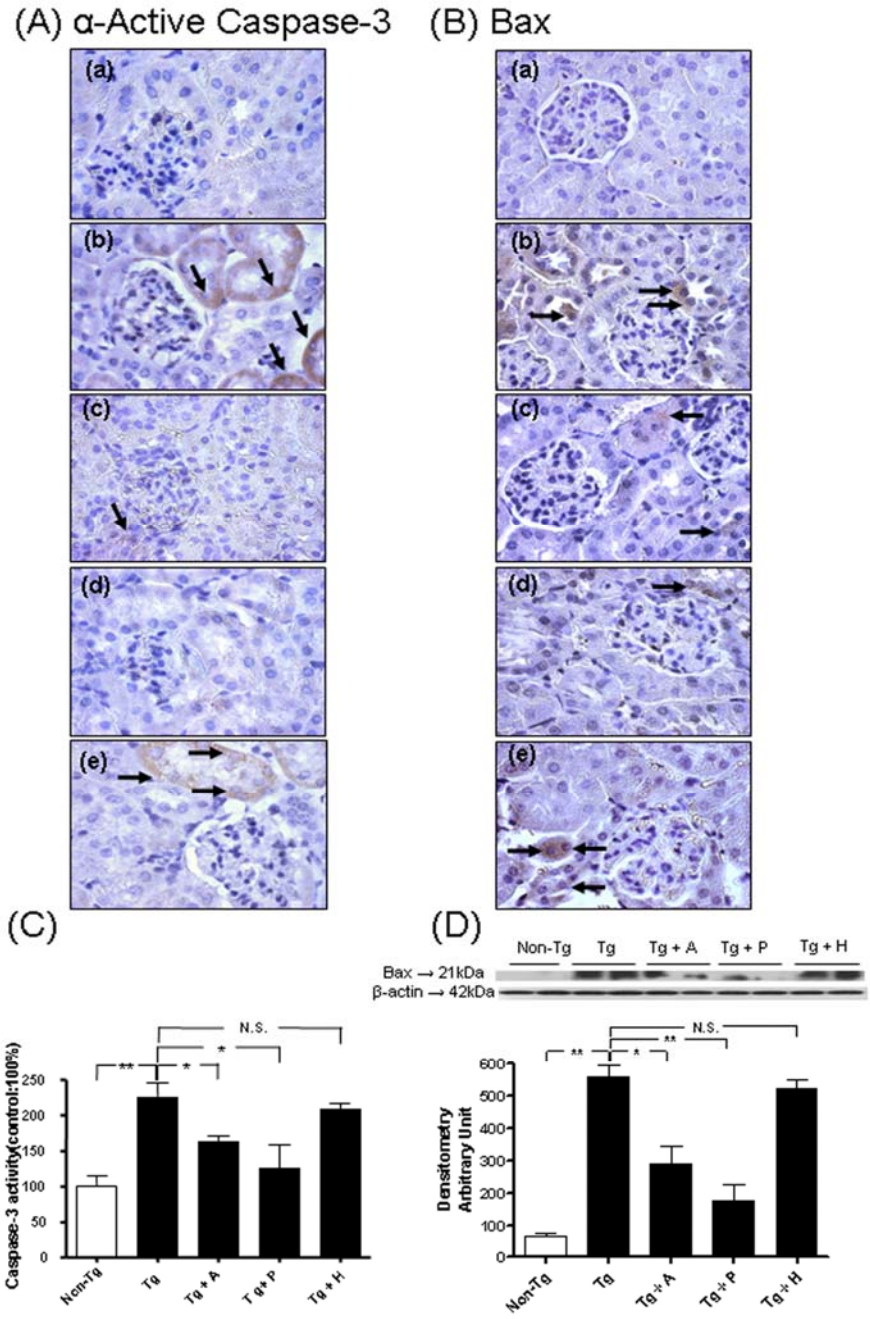


Figure 3-5. Immunohistochemical staining of α-active caspase-3 (A) and Bax (B) in male non-Tg and Tg mouse kidneys with or without apocynin, perindopril or hydralazine treatment, employing rabbit anti-α-active caspase-3 and anti-Bax antibodies (1: 50 dilution), respectively. (A and B) a, non-Tg control; b, Tg mouse; c, apocynin-treated Tg mouse; d, perindopril-treated Tg mouse; e, hydralazine-treated Tg mouse. Magnification x600. Arrows indicate caspase-3-stained cells (A) or Bax-stained cells (B). (C) Caspase-3 activity in RPTs from male non-Tg and Tg mouse kidneys with or without apocynin, perindopril or hydralazine treatment. (D) Western blot analysis of Bax expression (antibody dilution 1:500) in mouse RPT extracts of male non-Tg and Tg mice. All data are expressed as means ± S.D., N=8 (*$p<0.05$, **$p<0.01$; N.S., not significant). Non-Tg (empty bars) and Tg (solid bars) mice. A, apocynin; P, perindopril; H, hydralazine.

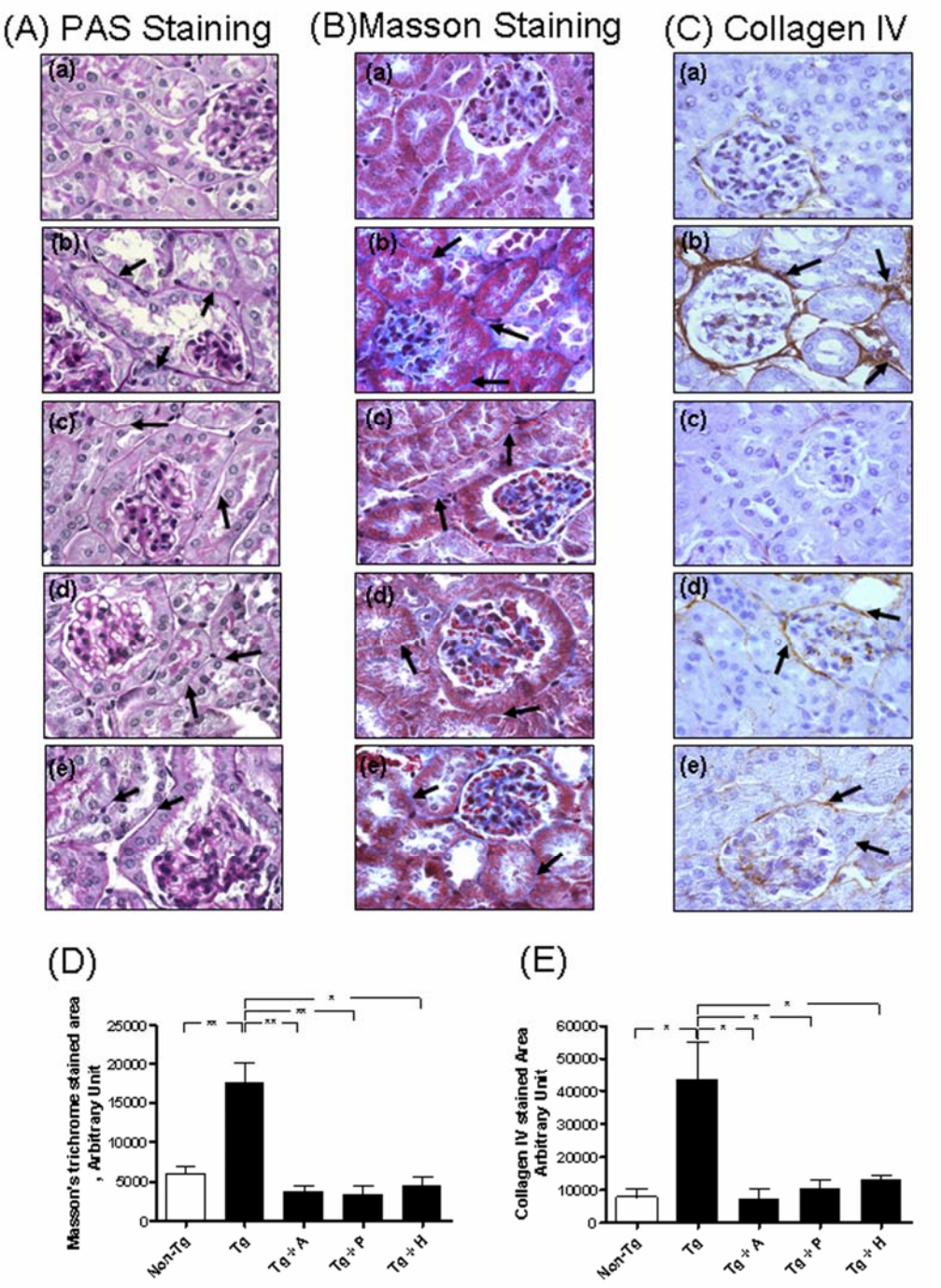


Figure 3-6. Periodic-acid Schiff (PAS) staining (A), Masson's Trichrome staining (B) and collagen type IV immunostaining (C) of male non-Tg and Tg mouse kidneys at week 20. (A), (B) and (C): a, non-Tg control littermate; b, Tg mouse; c, Tg mouse treated with apocynin; d, Tg mouse treated with perindopril; e, Tg mouse treated with hydralazine. Magnification x600. Arrows indicate location of extracellular matrix (A), collagenous materials (B) and collagen type IV immunostaining (C). Quantification of collagenous component accumulation (Masson staining) (D) and collagen IV deposition (E). Values are expressed as means ± S.D., N=8 in males (*$p<0.05$; **$p<0.01$; N.S., not significant). A, apocynin; P, perindopril; H, hydralazine.

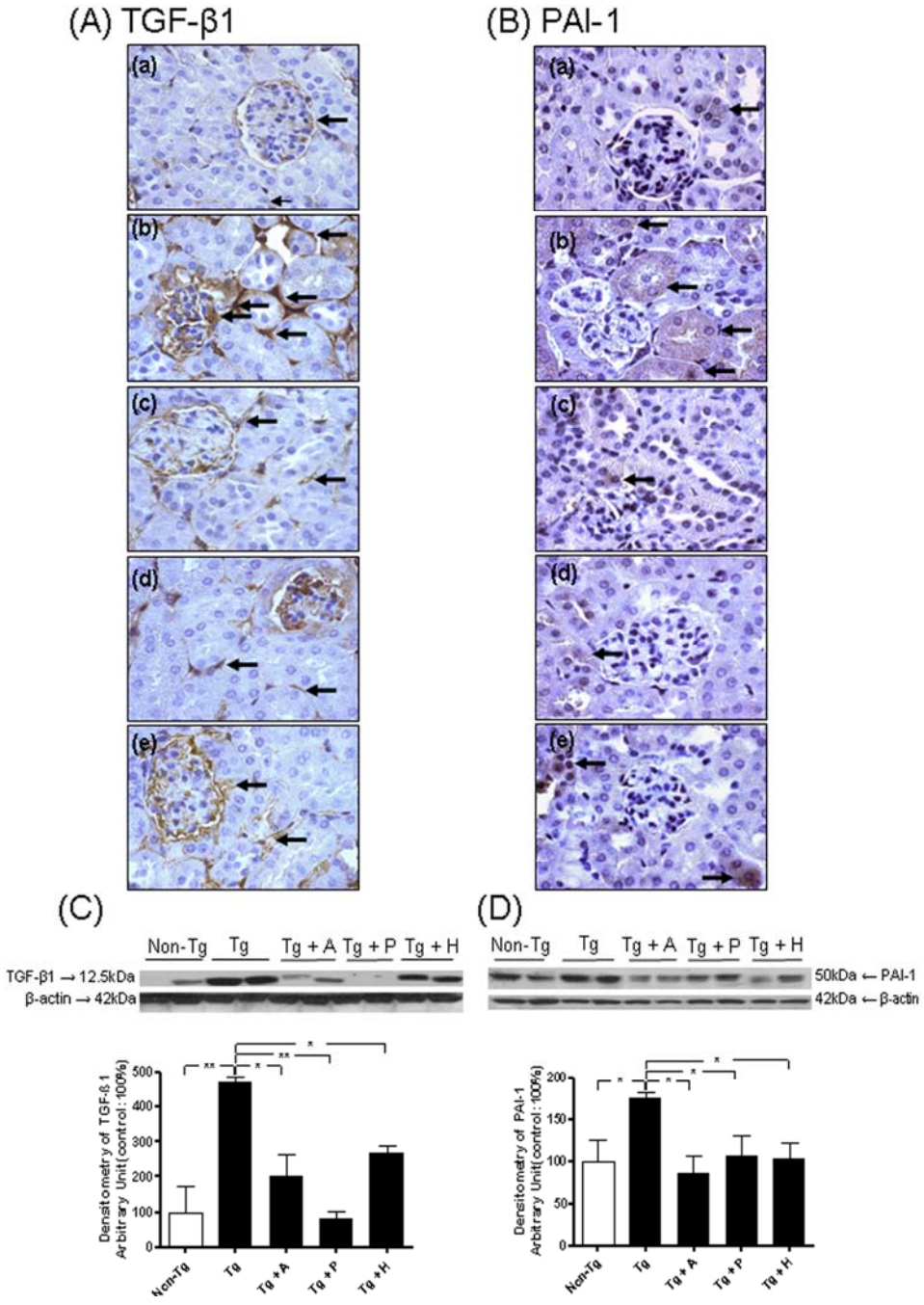


Figure 3-7. TGF-β1 (A) and PAI-1 (B) immunostaining of male non-Tg and Tg mouse kidneys at week 20. (A) and (B): a, non-Tg control littermate; b, Tg mouse; c, Tg mouse treated with apocynin; d, Tg mouse treated with perindopril; e, Tg mouse treated with hydralazine. Magnification x600. Arrows indicate TGF-β1 immuno-positive areas (A) or PAI-1 immuno-positive cells (B). Western blotting of TGF-β1 (C) and PAI-1 (D) expression in mouse RPTs. Values are expressed as means ± S.D., N=8 in males (*p<0.05; **p<0.01; N.S., not significant). A, apocynin; P, perindopril; H, hydralazine.

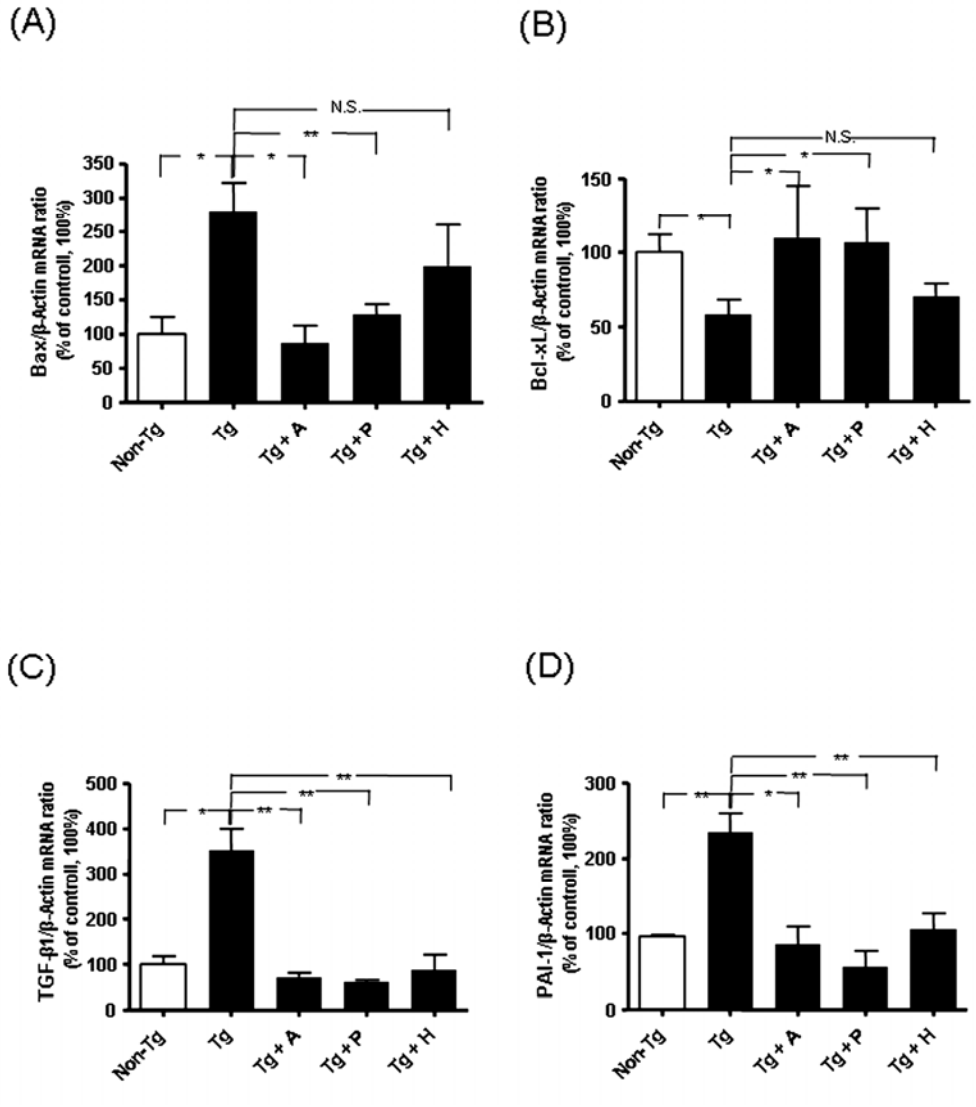


Figure 3-8. RT-qPCR assays of Bax (A), Bcl-xL (B), TGF-β1 (C) and PAI-1 (D) mRNA expression in RPTs of non-Tg and Tg mice with or without apocynin, perindopril or hydralazine treatment. Bax, Bcl-xL, TGF-β1, PAI-1 and β-actin mRNAs were run simultaneously in the same RT-qPCR assay. Bax, Bcl-xL, TGF-β1 and PAI-1 mRNA levels were normalized by corresponding β-actin mRNA levels. mRNA levels in non-diabetic non-Tg were considered as 100%. All data are expressed as means ± S.D., N=8 (*$p<0.05$, **$p<0.01$; N.S., not significant). Non-Tg (empty bars) and Tg (solid bars) mice. A, apocynin; P, perindopril; H, hydralazine.

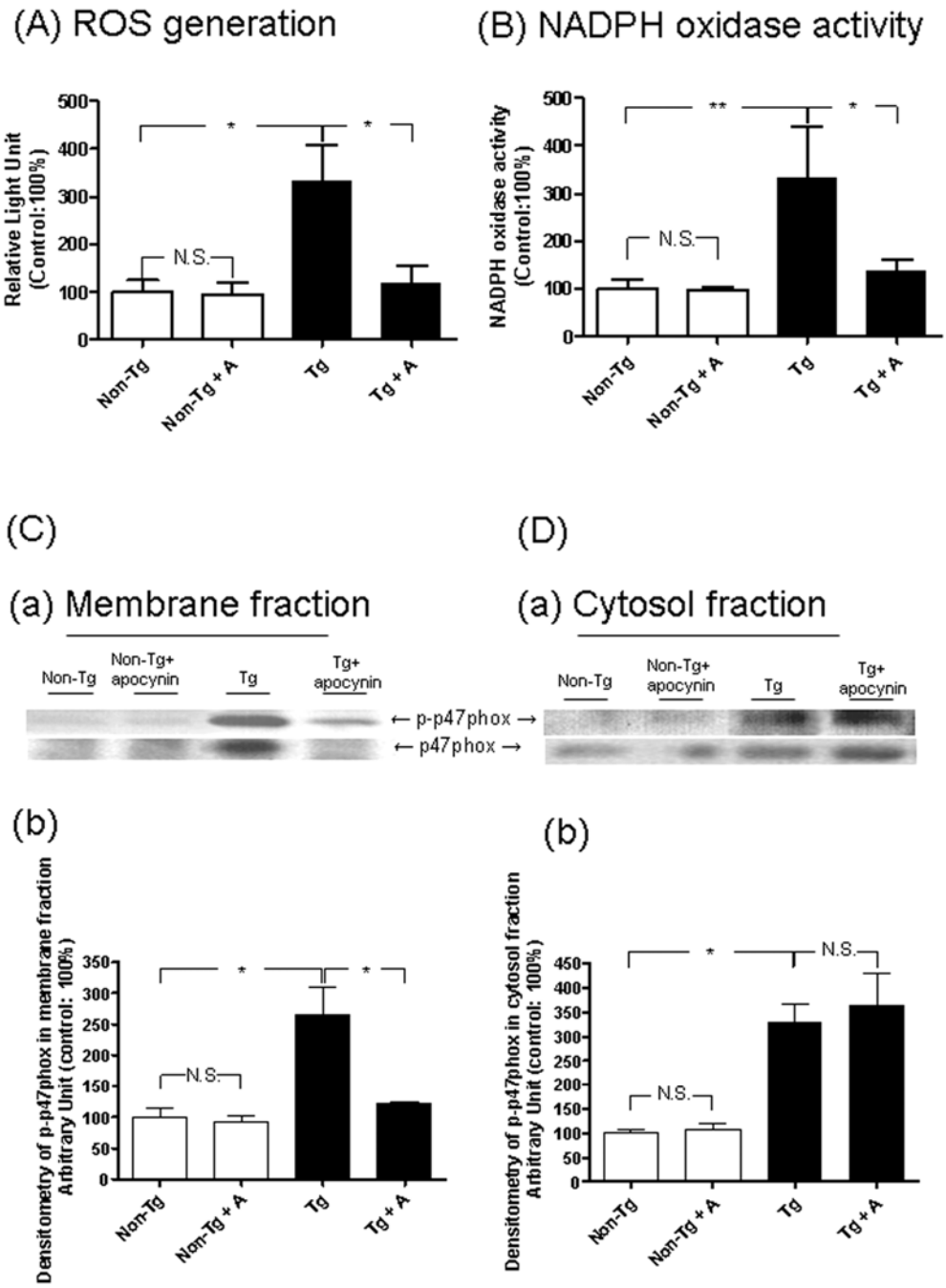


Figure 3-9. ROS generation, NADPH oxidase activity and Western blotting of p47phox in RPTs of non-Tg and Tg mice with or without apocynin treatment. (A) ROS generation; (B) NADPH oxidase activity; (C) Western blotting of p-p47phox and p47phox in membrane fractions of RPTs after immunoprecipitation with anti-p47phox. (a) Immunoblotting and (b) quantitation of p-p47 by densitometry. (D) Western blotting of p-p47phox and p47phox in the cytosolic fraction of RPTs after immunoprecipitation with anti-p47phox. (a) Immunoblot and (b) quantitation of p-p47 by densitometry. All data are expressed as means ± S.D., N=4 (*$p<0.05$; **$p<0.01$; ***$p<0.005$; N.S., not significant). Non-Tg (empty bars) and Tg (solid bars) mice. A, apocynin.

Chapter 4: Discussion

RAS activation and hyperglycaemia are 2 major risk factors for the development of DN. Although DN was traditionally considered to be primarily a glomerular disease, this contention has been challenged recently. Early tubular injury has been reported in DM patients with intact glomerular function. Evidence implicates the intrarenal RAS's contribution to DN. Since RPTCs express all RAS components, Tg mice overexpressing rAgt in their RPTCs were generated in our laboratory to investigate the role(s) of intrarenal RAS activation in renal injury. Previous studies from our laboratory have demonstrated the importance of the intrarenal RAS in renal damage and the induction of hypertension [313]. It remains to be seen whether intrarenal RAS activation alone could elicit microalbuminuria independently of systemic hypertension. Moreover, the effect of the intrarenal RAS in the diabetic condition deserves further experimentation. My PhD project focused on the influence of the intrarenal RAS on hypertension and kidney injury in non-diabetic and diabetic Tg mice overexpressing rAgt in their RPTCs in vivo and RPTCs overexpressing Agt in high glucose in vitro.

In our studies, non-diabetic Tg mice exhibited significant increases of SBP, albuminuria, RPTC apoptosis, and pro-apoptotic gene expression. Diabetic Tg mice showed a further increment of albuminuria, RPTC apoptosis, and pro-apoptotic gene expression, while their SBP was similar to that of non-diabetic Tg mice. RAS blockers and/or insulin treatment markedly attenuated these changes, except that insulin had no impact on hypertension. In vitro experiments indicated that the high-glucose milieu significantly increased apoptosis and caspase-3 activity in Agt-stable transfectants compared to control cells, and these alterations were inhibited by insulin and/or RAS blockers, as matched by the in vivo findings.

On the other hand, non-diabetic Tg mice showed significantly increased ROS production and NADPH oxidase activity as well as enhanced expression of TGF-β1, PAI-1, ECM proteins, collagen type IV, and NADPH oxidase subunit p47 in their RPTCs. Treatment with apocynin and perindopril ameliorated these changes, but apocynin had no effect on SBP. In contrast,

hydralazine prevented hypertension but not albuminuria, RPTC apoptosis and pro-apoptotic gene expression.

Our data indicate that intrarenal RAS activation and hyperglycaemia act in concert to induce albuminuria and RPTC apoptosis independently of systemic hypertension. ROS generation via NADPH oxidase activation mediates, at least in part, the intrarenal RAS effect on RPTC apoptosis, tubulointerstitial fibrosis and albuminuria in Tg mice.

Furthermore, in another on-going experiment, to avoid the nephrotoxicity of STZ and further delineate the outcome of intrarenal RAS activation, Tg mice overexpressing rAgt in their RPTCs were crossbred with Akita mice, a spontaneous T1D model, to generate Akita-rAgt-Tg mice. Preliminary data indicate that hyperglycaemia and intrarenal RAS activation caused ER stress in RPTCs *in vivo,* and the ER stress pathway contributed to RPTC apoptosis in diabetes, at least in the Akita model. RAS blockade was effective in attenuating some parameters of renal injury in Akita-rAgt-Tg mice.

4.1 Hypertension and albuminuria in rAgt-Tg mice

In our studies, rAgt Tg mice showed a 15-17 mmHg increase in BP (Figure 3-2, page 119) and a higher (4-5-fold greater) albumin/creatinine ratio (Figure 3-1, page 118), emphasizing the hypertensive role of intrarenal Agt in PTs. These BP results are consistent with the observations of others using the KAP2 promoter to target hAgt and hRen expression in RPTCs of Tg mice [5][309] [310] [389] [390] [391]. rAgt protein was readily detectable in RPTCs of Tg mice but only weakly in RPTCs of non-Tg mice (Figure 2-1, page 85). The rAgt transgene expression is driven by testosterone. We previously documented that testosterone administration (testosterone implant) increased the BP in female rAgt-Tg mice [313] unequivocally demonstrating a functional relationship between intrarenal Agt gene expression and BP development. Importantly, we did not find rAgt-HA fusion protein in plasma, indicating it was unlikely that this protein expressed in RPTCs of Tg mice had leaked into the circulation to induce hypertension [313]. Therefore, hypertension in Tg mice

is probably evoked by overexpressed intrarenal Agt that is converted to AngI by renin in RPTCs, which is then converted to AngII by ACE1 on RPTC membranes. It is also conceivable that renal AngII might exert a vasoconstrictive effect on glomerular arterioles via AT1R. Moreover, AngII may elicit hypertension through changes in sodium or fluid homeostasis, possibly via alterations in transport mechanisms in the kidneys. In fact, it has been demonstrated that AT1R activation by AngII in PTs stimulates the apical sodium-hydrogen exchanger [392] and augments epithelial sodium channel activity in the collecting ducts [393] [394]. Hence, AT1R activation by AngII in the kidneys modulates BP and fluid homeostasis. Finally, intrarenal RAS dysregulation has been implicated in certain models of hypertension [395] [396] [397].

Microalbuminuria is a gold standard marker of kidney dysfunction [398, 399]. Albuminuria can occur whenever a component of the glomerular filtration barrier (i.e., glomerular epithelia, GBM and podocytes) is injured. Although the glomerular endothelium is highly fenestrated, it can still hinder protein permeability [400] [401]. Widespread endothelial dysfunction is believed to result in proteinuria [402]. In diabetes, the GBM thickens, becomes more porous to proteins [403], and incurs a loss of charge-selectivity, further contributing to proteinuria [404]. The last member of the glomerular filtration barrier is the podocyte. Both human and animal studies have reported decreases in podocyte number, broadening of podocyte foot processes, and reductions of nephrin expression in diabetes [405] [406] [407] [408] [409]. Morphological damage to podocytes is believed to cause proteinuria. Thus, defects in the glomerular filtration barrier could result in elevated protein concentrations in the ultrafiltrate that could exceed the tubule's reabsorptive capacity and manifest as albuminuria, and even proteinuria. All these factors may contribute to the albuminuria seen in diabetic mice in the present experiments. On the other hand, an elevated albumin/creatinine ratio was observed in non-diabetic rAgt Tg mice [313] [251], a novel finding (Figure 3-1, page 118). Most studies have focused on

glomeruli, which were thought to be the source of albuminuria and proteinuria. The fact that the RPT is responsible for protein reabsorption in the ultrafiltrate and can contribute to albuminuria has been largely ignored. Enhanced ROS generation in RPTCs of rAgt Tg mice could cause RPTC dysfunction. Thus, deficient protein reabsorption by RPTCs may account for the microalbuminuria encountered in Tg mice. Moreover, the effect of locally-generated AngII on glomeruli may cause haemodynamic glomerular alterations which could further affect the GFR, subsequently impacting renal function. Therefore, the albuminuria found in diabetic rAgt Tg mice may be due to a combination of hyperglycaemia and intrarenal RAS activation, resulting in glomerular and RPTC dysfunction. Our data highlight the importance of intrarenal RAS activation in microalbuminuria development.

4.2 Relationship between hypertension and ROS

4.2.1 Animal experiments on hypertension and ROS

We noted elevated BP in rAgt Tg mice (Figure 3-2, page 119), indicating the effect of intrarenal RAS activation. Although detailed mechanism(s) by which intrarenal RAS activation induces hypertension are far from clear, we hypothesize that AngII generated from Agt in RPTCs may impact SBP through direct vasoconstriction of glomerular arterioles via the AT1R of AngII independently of ROS generation. Thus, hydralazine treatment of rAgt Tg mice normalized BP but ROS generation was not significantly decreased (Figure 3-2, page 119; Figure 3-1A, page 118).

It is unclear whether ROS could initiate hypertension or whether hypertension could result in increased ROS generation. If oxidative stress is the cause of hypertension, then antioxidants should be able to reduce oxidative damage and subsequently lower BP. Indeed, evidence indicates that while oxidative stress can elicit hypertension, hypertension can evoke oxidative stress as well. According to Tamara et al. [410], the effects of ROS or hypertension on each other can be summarized by the following 2 figures (Figures 4-1; 4-2 page 133): As illustrated, ROS could alter vascular tone,

while increases of laminar shear and oscillatory shear generated by hypertension could induce ROS generation via diverse pathways [410]. In the present study, ROS generation was analyzed by lucigenin assay. Heightened ROS generation was observed in rAgt Tg mice. Interestingly, apocynin attenuated renal ROS generation without affecting hypertension.

A study in Sprague-Dawley (SD) rats fed a high-salt diet for 4-5 weeks revealed an increase of oxidative stress with no such change in arterial pressure [411], suggesting that oxidative stress does not produce hypertension in this model. Nonetheless, experimental evidence indicates that oxidative stress causes hypertension. For example, Vaziri et al. demonstrated that BP elevation by lead was related to increment of the vasoconstrictor endothelin-3 generated in RPTCs and a decrease of the vasodilator nitric oxide (NO) [412]. The same group further found elevated ROS levels in the plasma and kidney cortex of lead-treated rats, while antioxidants lowered BP. In mice, deletion of the extracellular superoxide dismutase (SOD) gene caused an increase of oxidative stress and BP with low-dose AngII infusion [413]. On the other hand, transfer of the human extracellular SOD gene into spontaneously hypertensive rats (SHR) decreased BP [414]. Furthermore, augmented oxidative stress has been found in SHR, stroke-prone SHR, DOCA-salt hypertension and Dahl salt-sensitive rats [415] [416] [417] [418] [419] [420]. Various antioxidants, such as tempol, vitamins C and E, reduced the BP elevation in these animals, highlighting a role of oxidative stress in the etiology of hypertension.

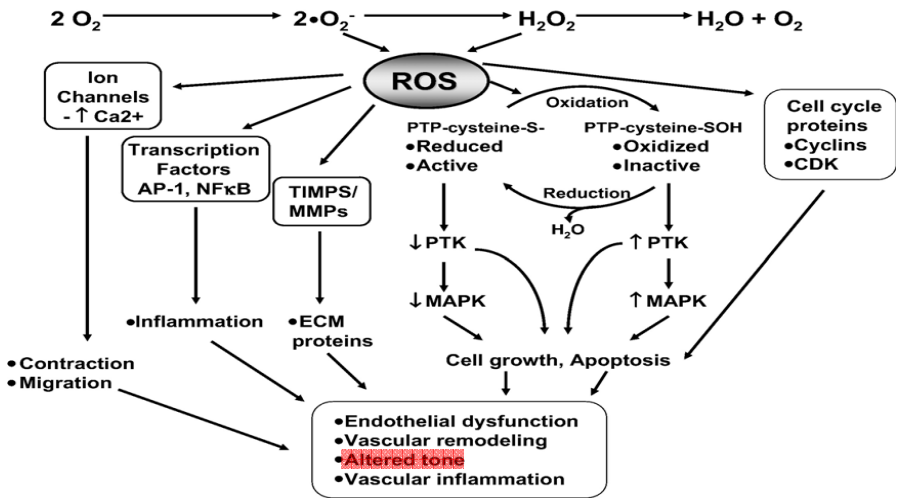


Figure 4-1. Intracellular ROS modify the activity of protein tyrosine kinases (PTK), such as Src, Ras, JAK2, Pyk2, PI3K, and EGFR, as well as MAPK, particularly p38MAPK, JNK and ERK5. These processes probably occur through oxidation/reduction of protein tyrosine phosphatases (PTP), which are susceptible to oxidation and inactivation by ROS. ROS also influence gene and protein expression by activating transcription factors, such as NF-κB, activator protein-1 (AP-1) and hypoxia-inducible factor-1 (HIF-1). ROS stimulate ion channels, such as plasma membrane Ca^{2+} and K^{+} channels, leading to changes in cation concentration. Activation of these redox-sensitive pathways results in numerous cellular responses which, if uncontrolled, could contribute to hypertensive vascular damage(modified from ref. [410])

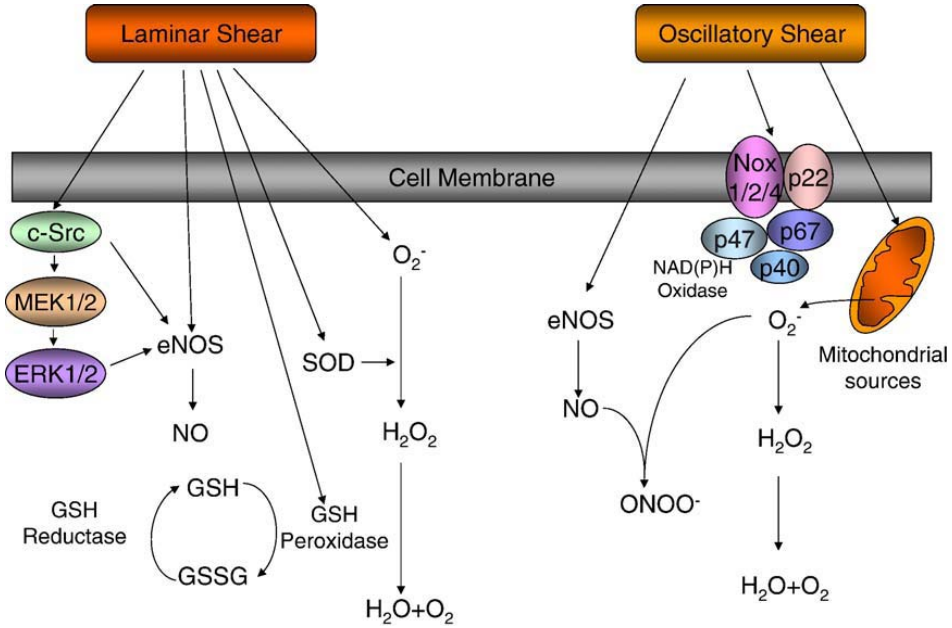


Figure 4-2. Mechanisms whereby mechanical forces influence generation of NO and ROS in vascular cells. Unidirectional laminar shear stress increases NO production by stimulating activation of endothelial nitric oxide synthase (eNOS) and by increasing eNOS mRNA and protein expression. These effects are mediated via c-Src-MAP kinase pathways. Laminar shear also increases expression/activity of SOD and glutathione peroxidase (GSH), thereby

contributing to antioxidant effects. Oscillatory shear causes a sustained increase in ROS production, derived in part from NADPH oxidase, xanthine oxidase, uncoupled eNOS and mitochondrial enzymes. Oscillatory shear-induced O_2^- formation interacts with NO to form peroxynitrite (ONOO–), which under physiological conditions is vascular protective, but under pathological conditions contributes to oxidative damage. GSH, glutathione; GSSG, GSH disulfide (ref. [410])

4.2.2 Clinical studies of antioxidants in the treatment of hypertension

Besides animal experiments on hypertension and ROS, clinical studies show that hypertension and ROS can influence each other. Humans with essential hypertension have a decreased antioxidant capacity [421] [422] and increased oxidative stress [423] [424]. Moreover, the Dietary Approaches to Stop Hypertension (DASH) trial, which examined the effects of a diet rich in fruit and vegetables compared to a combination diet rich in fruits, vegetables, low-fat dairy, reduced fat, and increased protein and fiber intake, found that both diets reduced BP in hypertensive and normotensive subjects more than a control diet [425]. Another study, which was a primary care intervention lasting 6 months, aiming to augment fruit and vegetable intake to 5 servings a day in hypertensive subjects, revealed not only increases in α - and β - carotene, lutein, β- cryptoxanthin, and vitamin C, but also decreases in SBP and diastolic blood pressure (DBP) [426]. These trials indicate that natural antioxidants from the diet can balance oxidative stress and can, therefore, lower BP.

Several clinical investigations have explored the use of a combination antioxidant supplement rather than dietary incorporation. However, these results were not consistent with those of the diet-control studies. For instance, one of the largest trials, undertaken by the Heart Protection Collaborative Group, observed no improvement in BP after treatment with a combination of ascorbic acid, synthetic vitamin E, and β-carotene versus placebo after 5 years in subjects thought to be at high risk for cardiovascular disease [427]. According to Ceriello et al. [428], one possible explanation is that there was a mix of antioxidants in the diet and that these antioxidants worked as a continuous chain, while antioxidant supplementation was usually given as only 1 or 2 substances. Therefore, the antioxidant chain was not

completely available. Moreover, if, after scavenging free radicals, an antioxidant is not restored by a following antioxidant in the chain, it could convert to a pro-oxidant. Therefore, antioxidant supplementation would have no effect or could even be damaging [428].

On the other hand, clinical studies have confirmed the impact of different types of antihypertensive treatments on oxidative stress [429] [430] [431] [432] [433] [434] [435]. All these trials showed that antihypertensive drug therapies, in addition to lowering BP, decreased oxidative stress. Even in normotensive subjects, antihypertensive treatment reduced systemic oxidative stress [430]. These reports support a role for hypertension in oxidative stress. Therefore, it is reasonable to conclude that ROS and hypertension influence each another.

4.2.3 NADPH oxidase activation mainly involved in ROS generation in rAgt Tg mice

rAgt Tg mice were found to generate more ROS than control littermates, indicating that intrarenal RAS activation was the main initiator of ROS production in rAgt Tg mice (Figure 3-1 (A), page 118). NADPH oxidase is an important source of ROS in the body. In the current study, by immunoprecipitation, phosphorylated p47 was seen to translocate to the membrane from the cytosol (Figure 3-9 (C), page 126) in rAgt Tg mice, offering direct evidence of NADPH oxidase activation. NADPH oxidase activity assay was conducted to further confirm our findings. NADPH oxidase activity was significantly higher in rAgt Tg mice than in wild-type controls (Figure 3-9(B), page 126). Apocynin treatment effectively normalized ROS generated from rAgt Tg mice, supporting the hypothesis that the increased ROS in rAgt Tg mice was from NADPH oxidase activation by AngII generated by the intrarenal RAS. Although we did not assess MPO activity in RPTCs which is indispensible for apocynin activation, Porubsky et al. reported that MPO is detactable in RPTs [436]. Thus, apocynin was effective in inactivating NADPH oxidase in RPTCs of rAgt Tg mice, preventing ROS generation and

subsequent kidney damage (Figure 3-1(A), page 118; Figure 3-9(B), page 126). We did not, however, explore the detailed pathway(s) of NADPH oxidase activation by AngII. According to the literature, AngII can activate NADPH oxidase through several possible mechanisms, either through its effects on NADPH oxidase subunits and/or by modulating their phosphorylation. Several authors have found that AngII influences the synthesis of NADPH oxidase subunits at multiple levels [437] [438] [439] [440]. Johnson et al. [441] reported that AngII may actually regulate NADPH oxidase activity by stimulating subunit phosphorylation. Serine phosphorylation of p47phox is a critical step for cytoplasmic subunit complex formation and translocation. During oxidase activation, serine S359 and/or S370 of p47phox must first be phosphorylated, followed by S379 phosphorylation, allowing the cytosolic complex to translocate to cytochrome b558 (gp91phox and p22 phox) on the membrane. Finally, S303 and/or S304 are phosphorylated, endowing the oxidase with full catalytic activity.

In the present study, apocynin had no effect on BP, indicating that ameliorating oxidative stress did not lower BP in the rAgt Tg model. Although some experiments have demonstrated that apocynin can decrease BP in rats and mice [442] [443, 444], recent investigations showed that it does not affect BP in hypertensive animals. For instance, Thallas-Bonke et al. [445] did not observe a significant decline of BP in apocynin-treated diabetic rats. Another paper reported no BP change in apocynin-treated SHR [446]. The reasons for the discrepancy in apocynin's effect can be complicated and deserve further exploration. One possible reason may be the unavailability of MPO in some cell types. Cells such as vascular smooth muscle cells (VSMCs) [447] are deficient in MPO; therefore, apocynin does not inhibit oxidase in these cells.

To summarize, we postulate that AngII generated from intrarenal RAS directly exerts its vasoconstrictive function on glomerular arterioles to increase BP while also activating NADPH oxidase, causing oxidative stress. Therefore, inhibition of one of AngII's effects would not completely reverse the abnormalities. Supporting this view, treatment of rAgt Tg mice with

hydralazine, a vasodilator, lowered BP without significantly decreasing ROS generation.

4.3 Effect of hyperglycaemia and intrarenal RAS activation on renal injury

To investigate the possible additive or synergistic effect of hyperglycaemia and intrarenal RAS action on kidney injury, diabetes was produced in Tg mice with the pancreatic islet β-cell toxin STZ. However, diabetic Tg mice did not show higher BP compared to euglycaemic Tg mice at 4 weeks after STZ-induced diabetes (Figure 2-3, page 87). On the other hand, changes in renal function and morphology were more severe in diabetic Tg mice than in both non-Tg diabetic and non-diabetic Tg mice (Figure 2-2; 2-4, page 86;88) These data indicate an additive effect of hyperglycaemia and the intrarenal RAS on renal injury.

4.3.1 Pathway(s) involved in RPTC apoptosis in non-diabetic and diabetic rAgt Tg mice

In the present study, RPTC apoptosis was detected in non-diabetic rAgt Tg mice by more than 1 method, such as TUNEL and caspase-3 activity assays, trying to compensate for their limitations. An increase of the pro-apoptotic gene Bax was further observed by immunohistochemistry and Western blotting, validating apoptosis in rAgt Tg mice, since Bax is one of most portent pro-apoptotic gene. According to other groups, TUNEL staining cannot always distinguish apoptotic and necrotic cells [448] [449] and this technique sometimes falsely identifies cells in the process of DNA repair [450]. Additional experiments have also demonstrated that cells in active gene transcription can yield false positive TUNEL results [451]. Therefore, besides TUNEL, we examined caspase-3 activity in isolated RPTCs from rAgt Tg mice, which was more precise in quantification, although there was tremendous overlap in the substrate preferences of the members of the caspase family, affecting the specificity of the assay [452]. Since high

expression of pro-apoptotic genes is another indicator of apoptosis, pro-apoptotic gene Bax expression was examined in rAgt-Tg mice at both the mRNA and protein levels. The anti-apoptotic gene Bcl-xL was also quantified and shown to be decreased in rAgt-Tg mice, which offered additional evidence of apoptosis. These data demonstrated that AngII generated from intrarenal RAS caused RPTC apoptosis. Indeed, our data are consistent with the studies of Cao et al. [453] and Aizawa et al. [454] showing that AngII infusion in rats enhanced TUNEL-positive cells in PTs. Although the precise mechanism(s) of RPTC apoptosis by AngII remain(s) unclear, in vitro experiments by Bhaskaran et al. [455] disclosed that AngII-induced tubular cell apoptosis may be mediated through TGF-β1, based on the fact that TGF-β1 promoted tubular cell apoptosis, while anti-TGFβ1 antibody attenuated the effect of AngII. This group also demonstrated that AngII caused RPTC apoptosis through the generation of ROS, p38 MAPK phosphorylation, expression of Fas, FasL and Bax, and activation of caspase 3, while Bcl-2 decreased in AngII-treated cells. Consistently, our rAgt Tg mice expressed more TGF-β1 in RPTCs, heightened caspase-3 and Bax expression as well as elevated ROS generation (Figure 3-7(A),(C), page 124; Figure 3-5(A),(C), page 122). Finally, our in vitro experiments showed increased caspase-3 activity and apoptosis in IRPTCs overexpressing Agt in a high-glucose milieu (Figure 2-12, page 96). Thus, our in vitro data are consistent with those of Bhaskaran et al. [455]. Treating rAgt Tg mice with the ACE inhibitor perindopril attenuated apoptosis and reduced caspase-3 activity, confirming the apoptotic effect of AngII. Grishko et al. [456] investigated the apoptotic cascade initiated by AngII in neonatal cardiomyocytes and reported that it activated the tyrosine kinase c-Src through its AT1R, which stimulated phospholipase C, leading to DAG formation and PKC activation. Activated PKC would phosphorylate p47phox, eliciting conformational rearrangements, and translocation of the cytosolic subunits to the membrane [457], therefore, triggers NADPH oxidase to increase ROS generation, resulting in DNA damage via p53 phosphorylation at serine 15 and serine 20 by activated

protein kinases. Apoptosis occurs as a result of p53-mediated elevation of the Bax-to-Bcl-2 ratio and p53-dependent activation of the mitochondrial death pathway (Figure 4-3, page 133) [456].

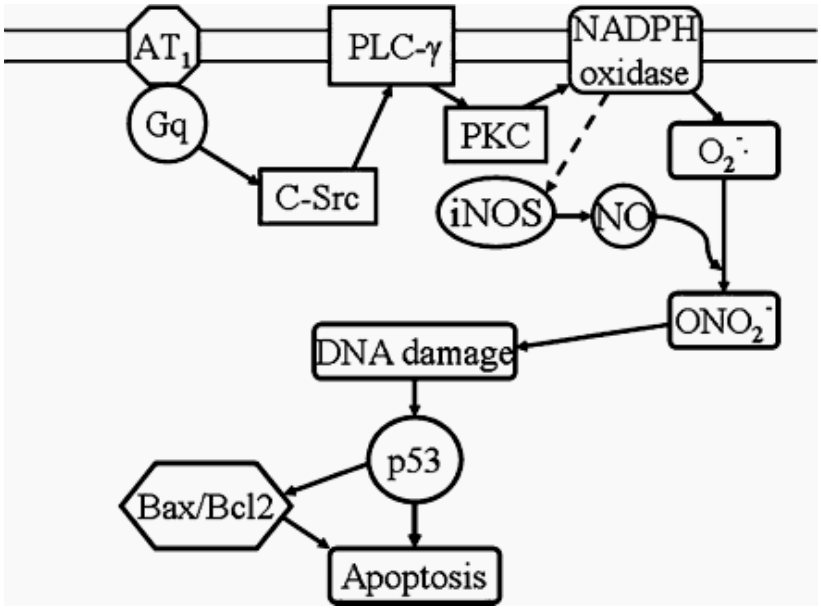


Figure 4-3. Scheme of Ang II-mediated apoptosis (ref. [456]).

According to Brownlee's hypothesis, oxidative stress is thought to be the common upstream element causing hyperglycaemia-induced damage [21]. The process of superoxide generation by hyperglycemia was discussed in the Introduction (page19). Thus, apoptosis in diabetic rAgt Tg mice can be a consequence of the additive effects of upstream ROS generated by hyperglycaemia and intrarenal RAS activation.

4.3.2 Tubulointerstitial fibrosis and RPTC hypertrophy in rAgt Tg mice

4.3.2.1 Evidence of tubulointerstitial fibrosis in rAgt Tg mice

In chronic renal disease, the decline of renal function correlates closely with the degree of tubulointerstitial fibrosis. TGF-β1 is a well-established

fibrogenic cytokine that stimulates the expression and accumulation of ECM in the kidneys [108]. In the current study, higher TGF-β1 expression was observed in the RPTCs of rAgt-Tg mice (Figure3-7, page 124), which could contribute to tubulointerstitial fibrosis in rAgt Tg mice from diverse aspects.

It is now proposed that (epithelial-mesenchymal transition) EMT plays a major role in the progression and maintenance of tubulointerstitial fibrosis. Yang and Liu [458] demonstrated that EMT is an orchestrated, highly-regulated process involving 4 key steps: (1) loss of epithelial cell adhesion, (2) de novo α-smooth muscle actin (α-SMA) expression and actin reorganization, (3) disruption of tubular basement membrane, and (4) enhanced cell migration and invasion into the interstitium. Much evidence suggests that renal tubuloepithelial cells can undergo EMT to become matrix-producing fibroblasts under pathological conditions and, therefore, participate in the pathogenesis of CKD. Cytokines, growth factors, adhesion molecules, and changes in ECM composition are involved in EMT [458] [459] [460]. Among these, TGF-β has been described as the most potent inducer of fibrosis and EMT [459] [461] [462] [463]. Moreover, in cultured tubuloepithelial cells, AngII evokes α-SMA expression and mesenchymal features [464]. AngII infusion into rats causes tubular injury associated with the neoexpression of α-SMA and vimentin in renal interstitial cells [465] [466]. Therefore, it is reasonable to speculate that AngII is also involved in EMT in the kidneys. In a very recent paper, in HK2 cells and rats infused with AngII, Carvajal et al [467] reported that AngII activates the Smad pathway during EMT.

In the present study, we did not extensively investigate EMT in tubulointerstitial fibrosis. Nevertheless, since TGF-β1 is the principal factor promoting EMT in vitro, many authors have worked on the signaling pathways involved in TGF-β1-induced EMT. For example, TGFβ1 was found to elicit Smad2 phosphorylation in a tubular epithelial cell line, and overexpression of an inhibitory Smad protein, Smad7, markedly inhibited TGFβ-evoked Smad2

activation, thereby preventing EMT and collagen synthesis [468] [469]. On the other hand, under the influence of growth factors, such as TGF-β, resident fibroblasts and tubular epithelia induce basement membrane-degrading enzymes, such as matrix metalloproteinase (MMP)-2 and MMP-9 [470]. Degradation of the tubular basement membrane results in disruption of tubular nephrons, and delaminated epithelial cells either fall off into the tubular fluid or migrate towards the interstitium under the influence of increasing growth factor gradients and chemoattractants [471]. Taken together, the pathological changes − obvious tubulointerstitial fibrosis in Tg mice overexpressing rAgt in their RPTCs − may be a partial consequence of higher level Agt generation, resulting in AngII elevation, which further induces TGF-β1, contributing to EMT and then tubulointerstitial fibrosis. Although we have not examined EMT-relevant pathway(s) in the present study, further experiments are warranted to clarify TGF-β1's effects on tubulointerstitial fibrosis during EMT in the rAgt Tg model.

Generally, the amount and composition of ECM depends on a delicate balance between synthesis and degradation. Both increased matrix deposition and decreased degradation contribute to matrix accumulation [472, 473]. ECM degradation is largely controlled by the plasminogen activator (PA)/plasminogen/plasmin system. Plasmin is released as plasminogen from the liver and activated by PA [474] [475]. Plasmin is involved in ECM degradation by directly degrading matrix proteins, such as fibronectin, laminin, and type IV collagen. It can also convert inactive MMPs to active forms that degrade collagenous proteins [476] [477] [478]. PAI-1, being the principal inhibitor of PA, constitutes a powerful, negative regulatory system to control the formation and activity of plasmin. Increased PAI-1 and decreased PA and plasmin have been reported in many experimental and human glomerular diseases characterized by mesangial matrix accumulation [479] [480]. TGF-β1 is found to increase PAI-1 production in cultured mesangial and tubular cells. RAS components, such as renin and AngII, rapidly and dramatically stimulate PAI-1 expression by mechanisms both

independent of and dependent on TGF-β1 [481] [345]. In the current study, PAI-1 was shown to be highly expressed in RPTCs of rAgt Tg mice (Figure 3-7, page 124), concomitantly with TGF-β1 in Tg mice. Our further experiments confirmed the fibrogenic effect of TGF-β1 and PAI-1 since more type IV collagen had accumulated in the tubulointerstitium of rAgt Tg mice kidneys without significant increases in glomeruli. Masson's trichrome and PAS staining yielded similar results in that rAgt Tg mice had more ECM deposition in the tubulointerstitial space, while RAS blockers and NADPH oxidase inhibitors effectively decreased TGF-β1, PAI-1 and ECM accumulation, indicating a role for ROS in ECM synthesis. Our findings suggest that tubular injury occurs earlier than glomerular damage in rAgt Tg mice. Interestingly, hydralazine did not significantly reduce ROS from the RPTCs of rAgt Tg mice, but decreased TGF-β1 and PAI-1 expression examined by both Western blotting and immunohistochemistry. ECM deposition in rAgt Tg mice treated with hydralazine was also significantly diminished (Figure 3-6, page 123), implicating hypertension effects *per se* on ECM deposition. However, Shihab et al. [482] observed no influence of hydralazine on TGF-β1 and PAI-1 expression in cyclosporine-induced nephropathy. Furthermore, Nakamura et al. demonstrated that AT1R blockade reduced PAI-1 mRNA expression in the kidneys while hydralazine did not [483]. The reasons for this discrepancy are not clear at present. One possible explanation is that both studies focused on the whole kidney, whereas we concentrated on RPTCs. In a Tg rat model harbouring both human renin and Agt genes [484], which was characterized by AngII-induced end organ damage, including the kidneys, hydralazine treatment did not improve albuminuria and renal injury, similarly to our findings. In another animal model, Tsukuba mice overexpressing the human renin and Agt genes, Kai et al. [485] showed that BP reduction with hydralazine did not prevent nephropathy. Their results are consistent with ours in that the effects of AngII on the kidneys are more than BP elevation.

TGF-β1 and PAI-1 were not examined in diabetic rAgt Tg mice in the

current study. Nicholas et al. nonetheless reported a renoprotective outcome of PAI-1 deficiency in STZ-induced diabetes, significantly reducing albuminuria and renal fibronectin or collagen levels [486]. Thus, we anticipated that TGF-β1 and PAI-1 expression would be increased in our diabetic Agt Tg mice.

4.3.2.2 Evidence of RPTC hypertrophy and atrophy in rAgt-Tg mice

The RPTC volume of rAgt Tg mice was significantly greater than that of their control littermates (Figure 3-3, page 120), suggesting RPTC hypertrophy in Tg mice. Interestingly, RPTC atrophy was also observed. One possible explanation for the appearance of atrophic RPTCs was apoptosis; our data clearly demonstrated RPTC apoptosis in Agt Tg mice. Specifically, apoptosis was detected at glomerular-tubular junctions in rAgt Tg mice, where atrophic tubular cells were seen. Tubular atrophy has been found in various experimental models of nephropathy as well as in human renal diseases, such as DN. Apoptosis is one of the possible mechanisms of atrophic tubular cells, which may further cause tubule atrophy and even atubular glomeruli [487] [488] [489] [490] [491, 492]. Among these experiments, the work of Benigni et al. highlighted the importance of RAS blockade on glomerule-tubule disconnection and atrophy. Our current data also showed that rAgt Tg mice treated with RAS blocker and apocynin had less apoptotic tubular cells at glomerular-tubular junctions. Importantly, no apoptotic cells were detected in glomeruli, and there was no significant increase of ECM deposition in the glomeruli of rAgt Tg mice either, suggesting that RPTC dysfunction occurs before glomerular changes. The present study, together with those of others, demonstrated that tubulointerstitial injury, characterized by interstitial fibrosis and tubular atrophy, may be a better predictor of renal disease than glomerular pathology, though the progression of chronic renal failure can be initiated by glomerular injury [388] [387].

Besides atrophic tubular cells, a significantly larger volume of RPTCs was apparent. One possible reason for the coexistence of hypertrophy and

atrophy in the kidneys of rAgt Tg mice may be the different levels of ROS and different ROS components, resulting in different consequences: low cellular ROS levels may induce cell hypertrophy, whereas high ROS levels could evoke cell apoptosis. Li et al. [493] have shown that ROS are able to stimulate both hypertrophy and apoptosis in rat VSMCs, depending on cellular ROS levels and their molecular species. Our data revealed that both hypertrophy and apoptosis were apparent in RPTCs of diabetic and rAgt Tg mice. *In vivo,* however, events leading to cell-cycle arrest and apoptosis may be more complex. According to David A. Allen's view, high glucose may simultaneously induce hypertrophy and apoptosis in the kidneys through diverse pathways which can interact with each other. Therefore, increased cellular hypertrophy and apoptosis may both occur after exposure to high glucose [494].

4.4 Limitations of the present study

We used a Tg mouse model which overexpresses rAgt in their RPTCs − to mimic intrarenal RAS activation. Since Agt is the sole substrate of the RAS, and RPTCs express all RAS components, theoretically, more AngII should be generated and affect the kidneys. Higher Agt expression was detected in rAgt Tg mice kidneys at both the mRNA and protein levels, and data on rAgt Tg mice confirmed our hypothesis. However, because of equipment limitations, we did not manually measure AngII concentration in glomerular ultrafiltrates, or in tubular fluid from rAgt Tg mice. Yet, RAS blockade prevented the abnormality seen in rAgt Tg mice, providing evidence of increased AngII concentration in the kidneys. In fact, AngII concentration in the kidneys has been accurately measured since the early 1990s. Seikaly et al.[271] quantified Ang concentrations by RIA in glomerular filtrate, proximal tubular fluid and systemic plasma. They found that AngII in both glomerular filtrate and proximal tubular fluid was nanomolar, while AngII in systemic plasma was picomolar. Shortly thereafter, Braam et al. [272] reported similar AngII concentrations: 13 ± 2 nM in proximal tubular fluid and 155 ± 26 pM in

plasma.

Besides DN, hypertension is another common complication of diabetes, often concomitant with DN and aggravating renal damage. Nonetheless, STZ-induced diabetic mice did not develop hypertension, at least in the early stage. Thus, a diabetic animal model with hypertension would be better to replicate the human condition. Another obvious limitation is the toxic effect of STZ. In fact, Tay et al. [370] already demonstrated that even with careful optimization of the STZ dose, superimposed acute tubular necrosis was hard to separate from DN. A diabetic mouse model better than STZ-induction was necessary for further experiments. Therefore, a spontaneously diabetic model, the Akita mouse, was adopted in on-going experiments. However, Akita mice develop diabetes as early as age 3-4 weeks, when it is not realistic to begin measuring BP by tail-cuff because of their small body size. Thus, it is hard to distinguish if the higher BP measured from age 9-10 weeks is a consequence of diabetes development, or if it is concomitant with diabetes onset.

Chapter 5: Unpublished Data

Unpublished data to further explore the molecular mechanism(s) of the synergism of hyperglycaemia and intrarenal RAS activation in hypertension and nephropathy development in diabetes

Although STZ is a commonly-used chemical inducing diabetes in rodent models, its nephrotoxic effect is unavoidable. Hence, we studied Akita mice to dissect the synergism of hyperglycaemia and intrarenal RAS activation on nephropathy development in diabetes. Diabetes in Akita mice is caused by a mutation of the insulin 2 gene (*Ins2*) whose cysteine residue at the seventh amino acid of the A chain is replaced by tyrosine. This cysteine is involved in the formation of 1 of 2 disulfide bonds between the A and B chains. Disruption of the intramolecular disulfide bond induces a drastic conformational change in *ins2*. The futile synthesis and degradation of pro-insulin and accumulation of misfolded pro-insulin evoke overall dysfunction of β-cells, which causes hyperglycaemia [495]. Akita mice develop hyperglycaemia at age 3-4 weeks. This spontaneous diabetes model is an ideal substitute for STZ-induced diabetes.

5.1 Intrarenal RAS activation in Akita mice

No one has reported intrarenal RAS activation in Akita mice till now. Our unpublished data revealed, for the first time, intrarenal RAS activation in Akita mice (unpublished Figure 5-1). By immunohistochemistry, we have also comfirmed transgene rAgt expression in Akita-rAgt-Tg mice (data not shown).

5.2 Hypertension in Akita mice

Unlike STZ-diabetic mice, hypertension was observed in Akita mice at age 9-10 weeks, at a time when they were already diabetic for 5-6 weeks (unpublished Figure 5-2). Hypertension is a phenotype that is not seen in STZ-diabetic mice. From the viewpoint of BP, Akita mice could be a better model for

DN than STZ-diabetic mice. At present, the reason(s) why Akita mice are hypertensive are unclear. Gurley et al. [496] similarly reported that Akita mice developed hypertension whereas STZ-diabetic mice did not, but they provided no explanation for their finding. Genetic modification in Akita mice may be a possible reason for the hypertension occurring in them. However, more studies are needed to verify this hypothesis. Interestingly, our Akita-rAgt-Tg mice, which were generated by crossbreeding Akita mice with rAgt-Tg mice, displayed significantly higher BP than Akita mice (unpublished Figure 5-2), implicating an additive effect of hyperglycaemia and intrarenal RAS activation on hypertension development in Akita-rAgt-Tg mice. RAS blockers lowered blood pressure of Akita and Akita-rAgt-Tg mice (unpublished Figure 5-2).

5.3 Kidney damage in Akita mice

When Akita mice were euthanized at age 16 weeks, the ACR was measured by ELISA. Similarly to the observations on STZ-diabetic mice, the ACR in Akita mice was higher than in WT control mice; it was further augmented in Akita-rAgt-Tg mice (unpublished figure 5-3). RAS blockers were effective in decreasing albumin/creainine ratio of Akita and Akita-rAgt Tg mice. Akita-rAgt-Tg mice also had a higher kidney/body weight ratio than Akita mice, and the latter was already higher than in WT control mice (unpublished figure 5-3). RAS blockers, however, did not show effectiveness in reducing kidney/body weight ratio. Moreover, RAS blockers did not improve histological changes seen in Akita and Akita-rAgt Tg mice (unpublished Figure 5-4). Both Masson trichrome staining and collagen IV immunostaining indicated an increase in ECM deposition in Akita mice as compared to WT controls and further increased of ECM in Akita-rAgt-Tg mice kidneys compared to Akita mice (unpublished Figure 5-5). Higher expression of pro-fibrotic genes TGFβ1 and PAI-1 provided further evidence for ECM deposition in akita and Akita-rAgt Tg mice (unpublished Figure 5-10, A and B). Apoptosis was readily detected in Akita mice, whereas even more apoptotic PTCs were encountered in Akita-rAgt-Tg mice. In contrast to STZ-diabetes, apoptotic cells in the glomeruli were also noted in Akita and Akita-rAgt-Tg mice

(unpublished Figure 5-6). In addition, active caspase-3 was detected in RPTCs (unpublished Figure 5-6). Akita and Akita-rAgt-Tg mice also expressed more caspase-12 mRNA and protein in their RPTCs than non-diabetic mice (unpublished Figure 5-7). Caspase-12 has been demonstrated to contribute to apoptosis in response to ER stress [221]. When activated, caspase-12 translocates to the cytoplasm from the ER membrane and induces caspase 9, which subsequently cleave caspase-3 to generate active caspase 3. Although cytochrome c released from the mitochondria is believed to be required for caspase-9 activation during apoptosis [497] [498], caspase-9 as well as caspase-12 and -3 are stimulated in cytochrome c-free cytosol of murine myoblast cells under ER stress. These results suggest that caspase-12 can induce caspase-9 without cytochrome c involvement [222]. We have detected active caspase-9 in Akita and Akita-rAgt-Tg mice (unpublished figure 5-8). Other ER stress-associated genes, CHOP/GADD153 and GRP78, also showed a tendency to much higher expresssion in Akita-rAgt-Tg than in Akita mice (unpublished Figure 5-10, D and E). Thus, our unpublished data indicate that hyperglycaemia and intrarenal RAS activation may induce ER stress in RPTCs *in vivo* and contribute to RPTC apoptosis.

Preliminary data showed that RAS blockade mitigated some kidney abnormalities in Akita and Akita-rAgt-Tg mice, such as ER stress-associated apoptotic gene caspase-12, suggesting that RAS blockade normalized ER stress-relevant gene expression, which promisingly points to another novel effect of intrarenal RAS activation on ER stress. Thus, the mechanism(s) of intrarenal RAS on apoptosis can be very complicated, and further work is warranted. The perspective of this experiment is described in Chapter 6.

5.4 Unpublished data

Figure 5-1 to figure 5-10.

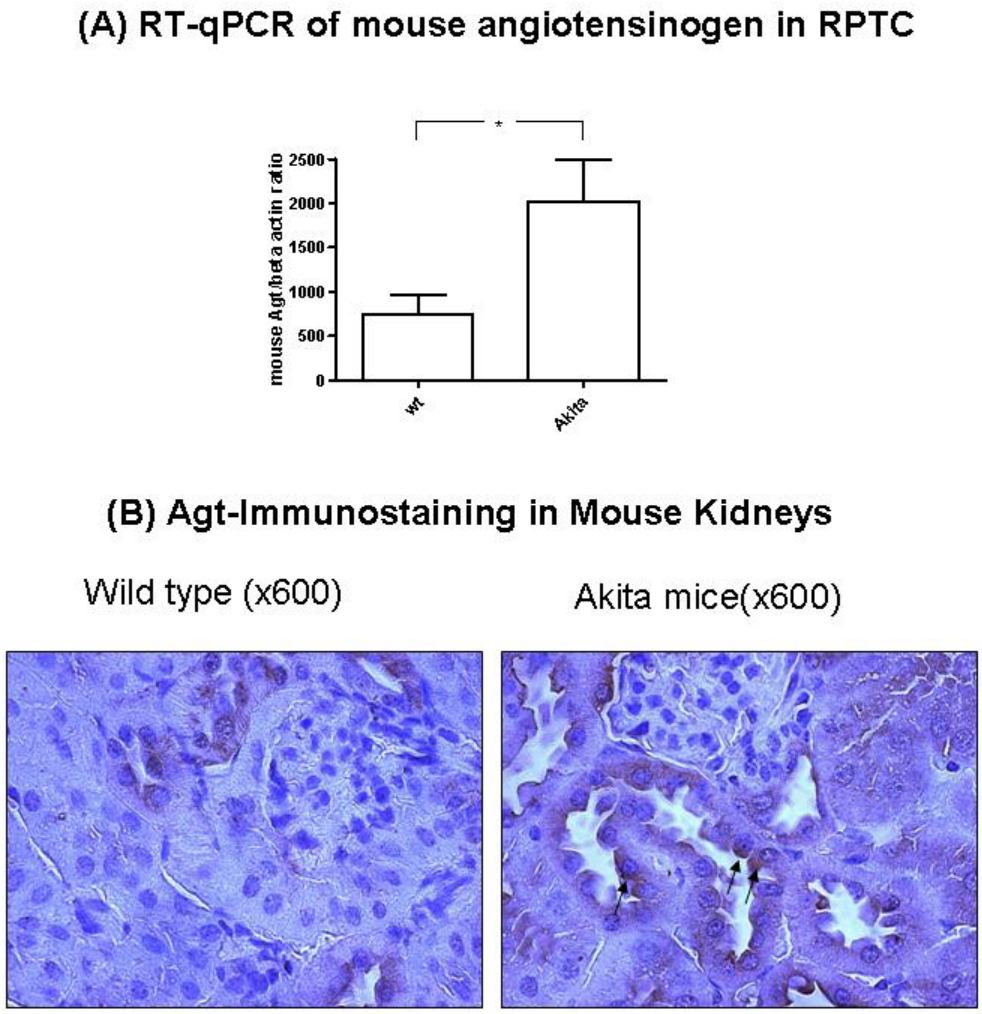
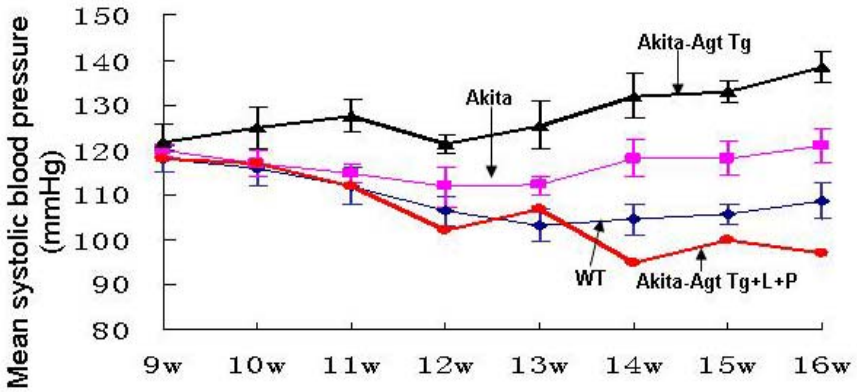


Figure 5-1. Agt expression in mouse kidneys. (A) RT-qPCR of mouse Agt in RPTC shows a higher expression of Agt in Akita mice. (B): Immunohistochemistry demonstrates more Agt staining in Akita RPTCs. *$p<0.05$.

(A)



(B)

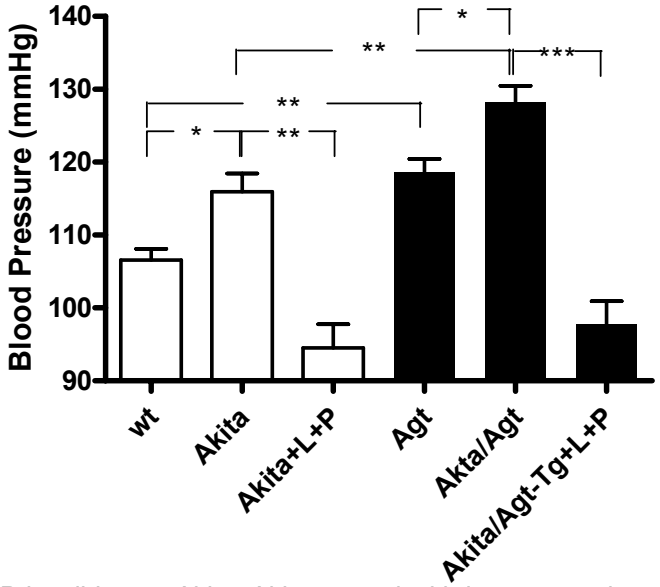
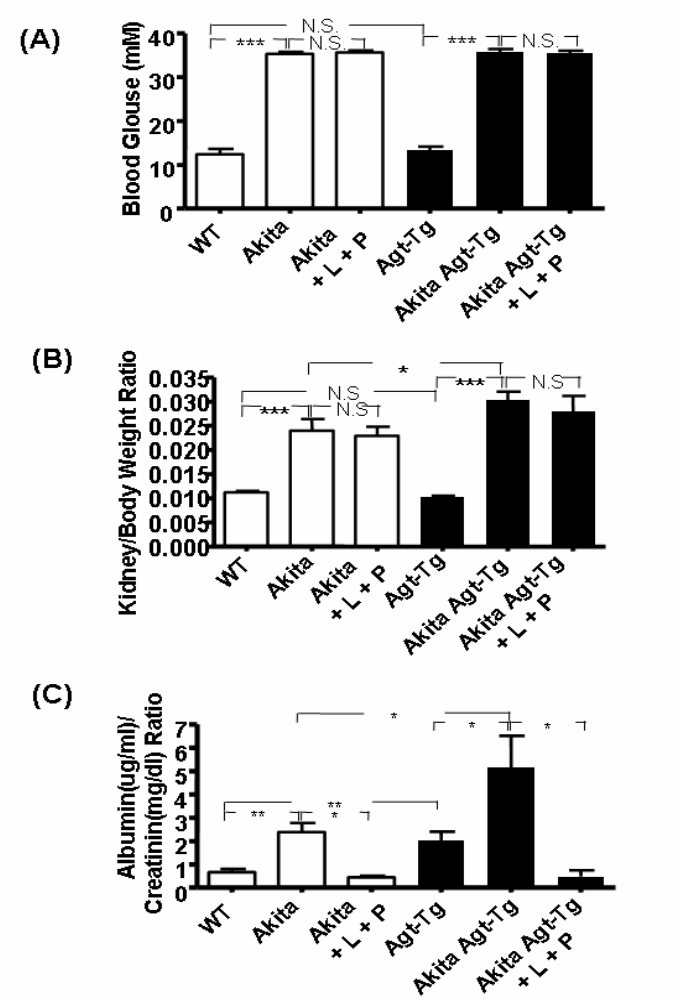


Figure 5-2. (A) SBP in wild type, Akita, Akita treated with losartan and perindorpril, Agt Tg mice, Akita-Agt Tg mice and Akita-Agt Tg mice treated with losartan and perindopril. (A) Longitudinal changes in mean SBP. Baseline SBP was measured in all mice for 5 days before the first SBP reading. (B) Cross-sectional analysis of SBP. All data are expressed as means ± S.D. (*$p<0.05$; **$p<0.01$; ***$p<0.005$;). L, losartan; P, perindopril; WT, wild type.



(D)

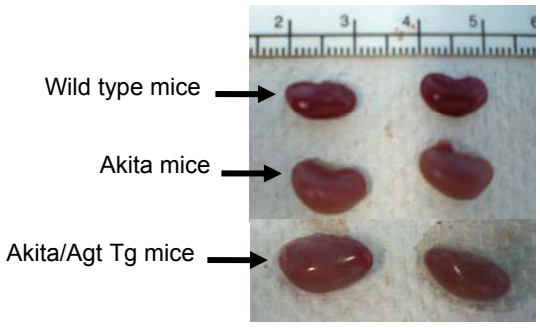


Figure 5-3. (A) Blood glucose level, (B) Kidney/body weight ratio, (C) albumin/creatinine ratio (D) kidney pictures in wild type, Akita, Akita treated with losartan and perindorpril, Agt Tg mice, Akita-Agt Tg mice and Akita-Agt Tg mice treated with losartan and perindopril. The kidney-to-body weight ratio was measured as the weight of 2 kidneys per body weight. L, losartan; P, perindopril; WT, wild type; *p<0.05; **p<0.01; ***p<0.005;N.S: not significant. All data are expressed as means ± S.D.

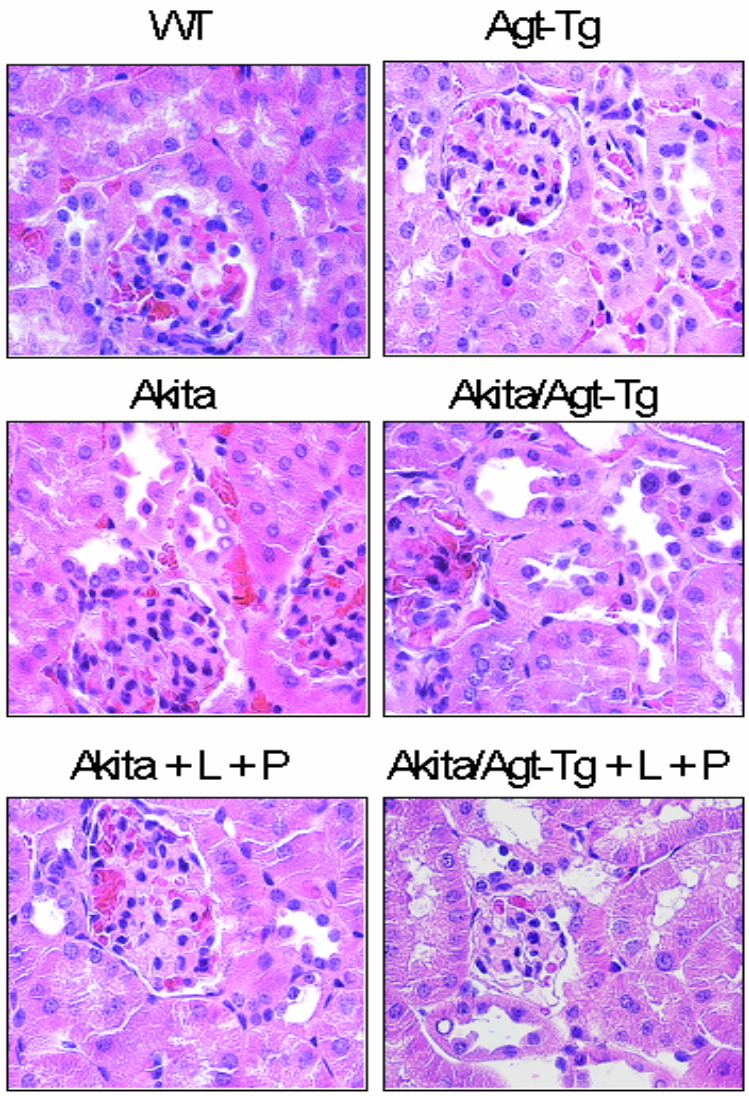


Figure 5-4. (A) HE staining of kidneys in wild type, Akita, Akita treated with losartan and perindorpril, Agt Tg mice, Akita-Agt Tg mice and Akita-Agt Tg mice treated with losartan and perindopril. L, losartan; P, perindopril; WT, wild type.

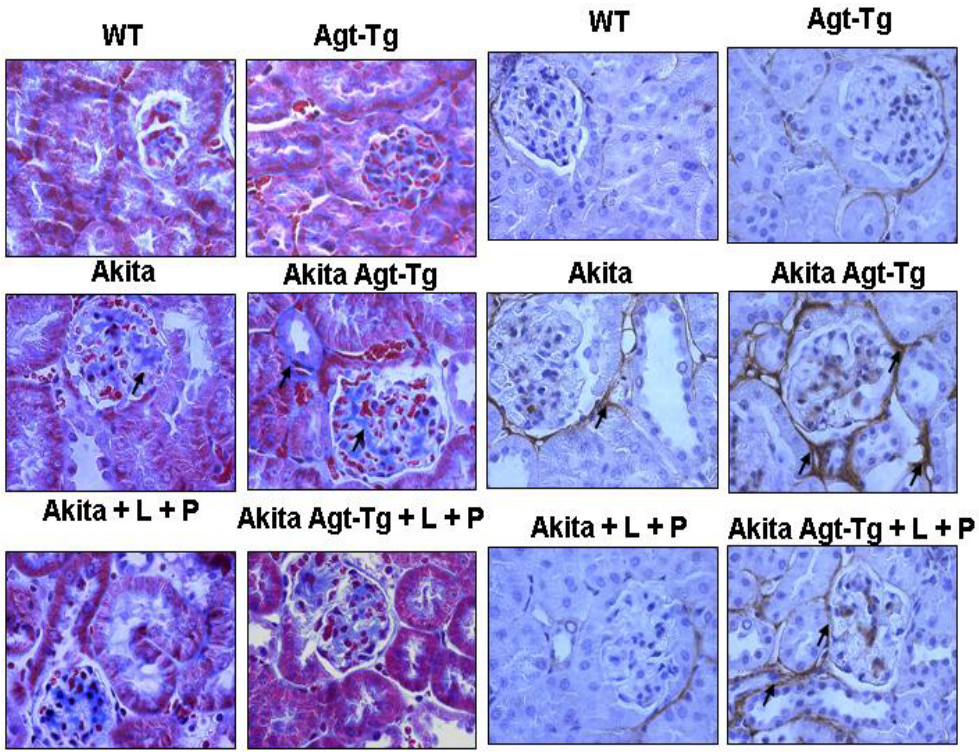


Figure 5-5. (A) Masson's Trichrome staining, (B) collagen type IV immunohistochemical staining of kidneys in wild type, Akita, Akita treated with losartan and perindorpril, Agt Tg mice, Akita-Agt Tg mice and Akita-Agt Tg mice treated with losartan and perindopril. L, losartan; P, perindopril; WT, wild type.

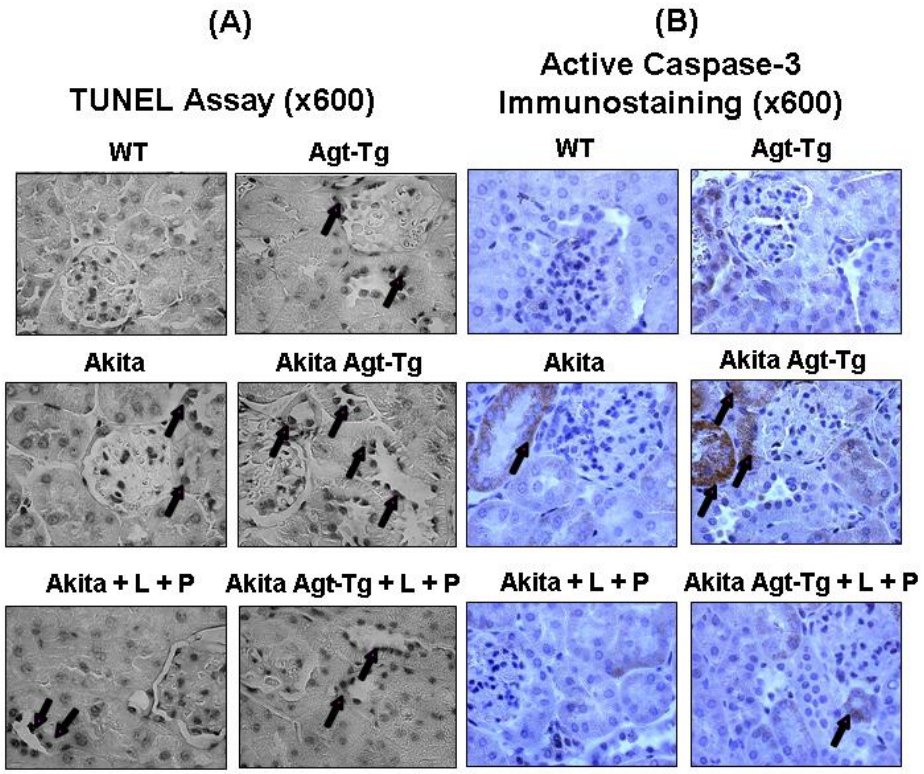


Figure 5-6. Apoptosis analyzed by TUNEL assay (A) and active caspase 3 immnunohistochemical staining (B) of kidneys in wild type, Akita, Akita treated with losartan and perindorpril, Agt Tg mice, Akita-Agt Tg mice and Akita-Agt Tg mice treated with losartan and perindopril. L, losartan; P, perindopril; WT, wild type.

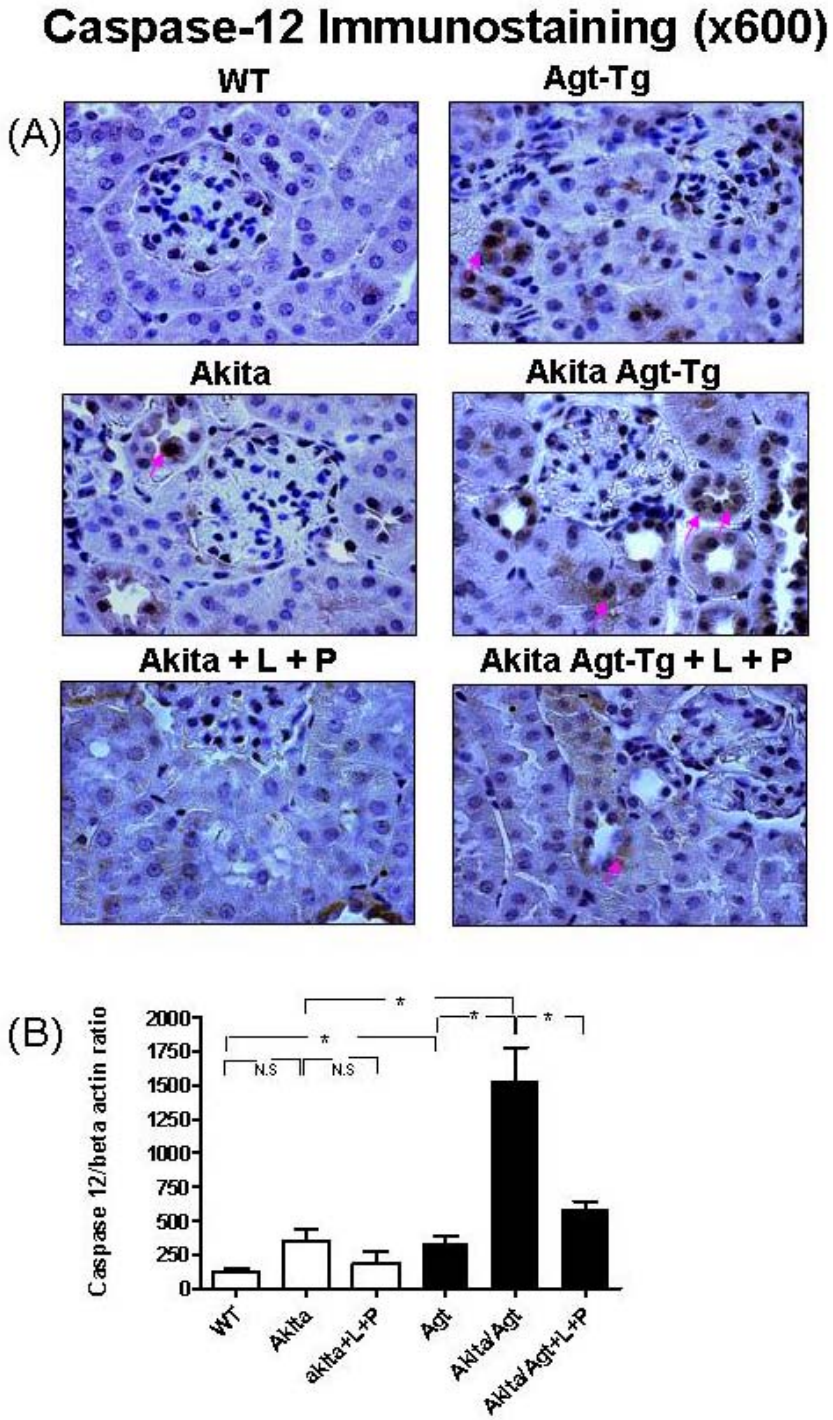


Figure 5-7. Caspase 12 expression in kidneys of wild type, Akita, Akita treated with losartan and perindorpril, Agt Tg mice, Akita-Agt Tg mice and Akita-Agt Tg mice treated with losartan and perindopril. (A): immunohistochemical staining of caspase 12. (B): RT-qPCR of caspase 12 mRNA expression. *p<0.05; N.S: not significan L, losartan; P, perindopril; WT, wild type.

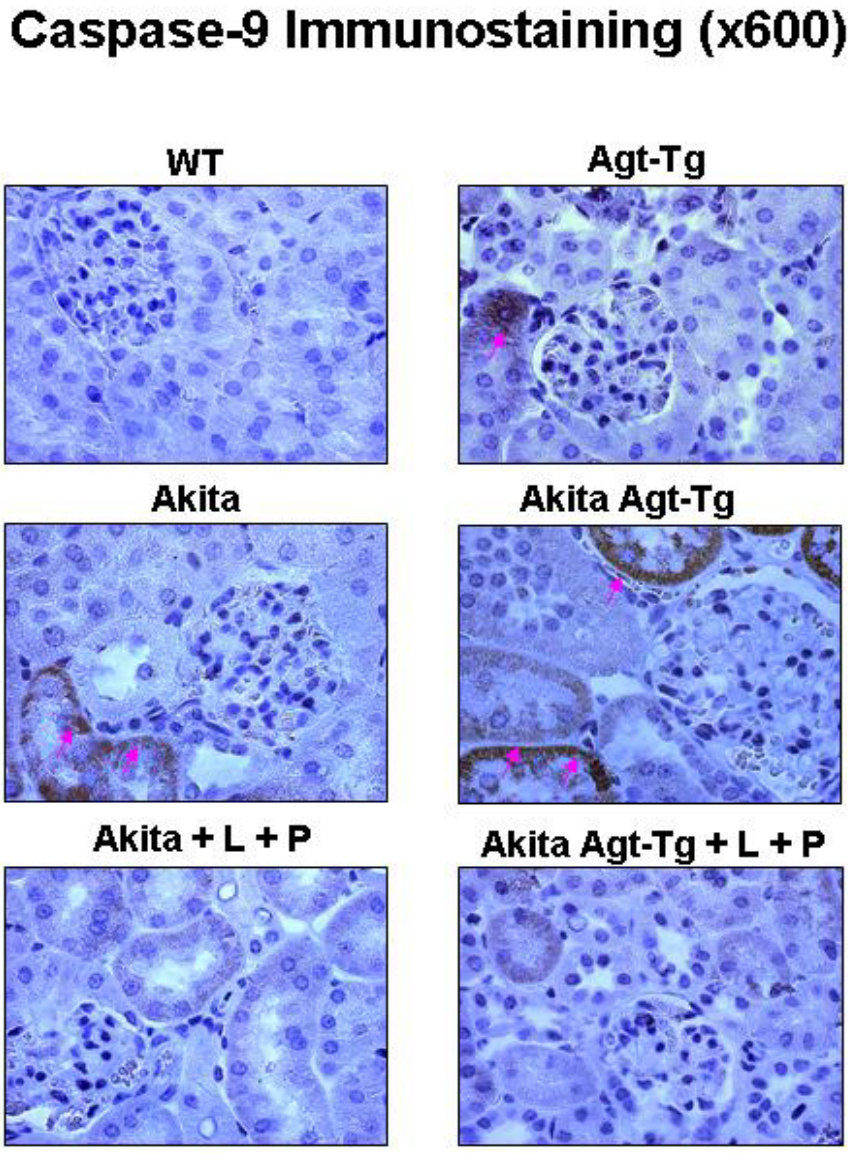


Figure 5-8. Immunohistochemical staining of active caspase-9 in kidneys of wild type, Akita, Akita treated with losartan and perindorpril, Agt Tg mice, Akita-Agt Tg mice and Akita-Agt Tg mice treated with losartan and perindopril. L, losartan; P, perindopril; WT, wild type.

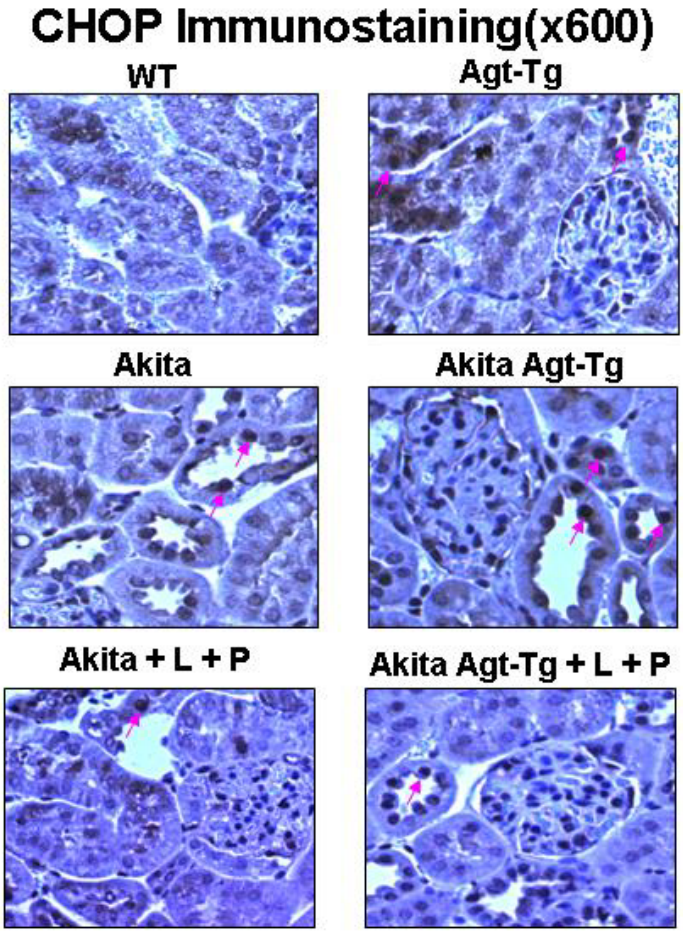


Figure 5-9. Immunohistochemical staining of CHOP in kidneys of wild type, Akita, Akita treated with losartan and perindorpril, Agt Tg mice, Akita-Agt Tg mice and Akita-Agt Tg mice treated with losartan and perindopril. L, losartan; P, perindopril; WT, wild type.

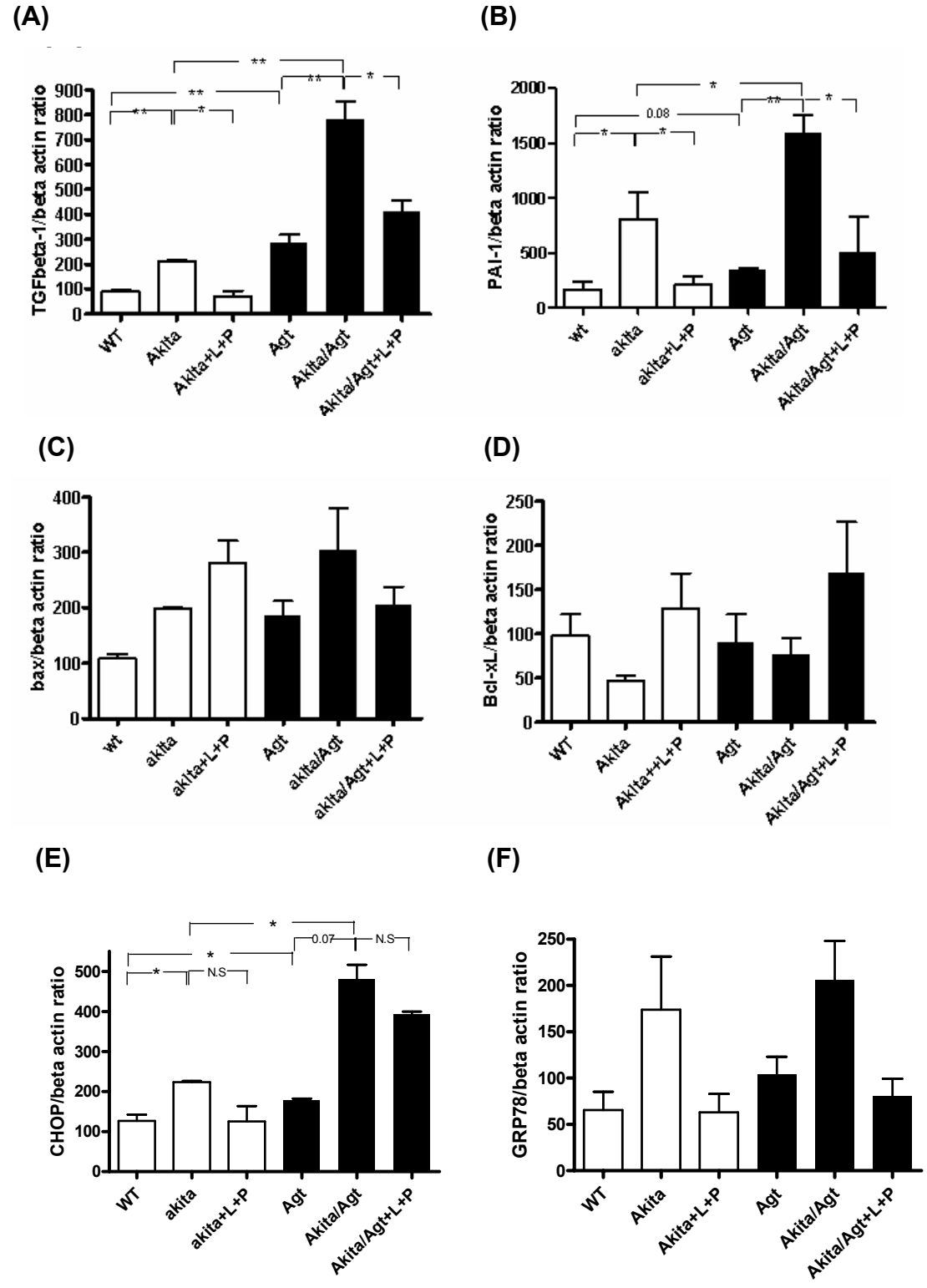


Figure 5-10. RT-qPCR of (A)TGF-β1, (B)PAI-1, (C) Bax, (D)Bcl-XI, (E)CHOP and (F)GRP78 mRNA expression in RPTs of kidneys of wild type, Akita, Akita treated with losartan and perindorpril, Agt Tg mice, Akita-Agt Tg mice and Akita-Agt Tg mice treated with losartan and perindopril. *p<0.05; **p<0.01;N.S:not significant. L, losartan; P, perindopril; WT, wild type

Chapter 6: Perspectives of Research

6.1 The study of Akita and Akita-Agt-Tg mice provided preliminary data on ER stress and RPTC apoptosis. However, more experiments are necessary to validate the role of hyperglycaemia and intrarenal RAS in ER stress leading to RPTC apoptosis

We propose the following future studies:

6.1.1 Effect of RAS blockers

To block intrarenal RAS activation, Akita and Akita-rAgt-Tg mice will be treated with RAS blockers, such as ACE inhibitors and/or ARBs. Investigations will be conducted to determine whether RAS blockers attenuate renal damage. Briefly, Akita and Akita-rAgt-Tg mice will be treated with perindopril (3 mg/kg) and losartan (30 mg/kg) in drinking water from age 10 weeks until 16 weeks, when they will be euthanized. BP will be measured from age 8 or 9 weeks until 16 weeks, 2-3 times per week. Urine will be collected every other week for albuminuria analysis by the ACR. After sacrifice, half of the kidneys will be fixed for histology, with hematoxylin-eosin (HE) and Masson's trichrome staining, immunohistochemistry, TUNEL assay, etc. RPTCs will be isolated from the remaining kidneys for RNA and protein extraction to detect the relative gene expression of caspase-12, CHOP, JNK, TGFβ1, collagen IV, etc., by RT-qPCR and Western blotting. It is anticipated that RAS inhibition will improve the renal histology and decrease the expression of pro-apoptotic genes, ER stress-related genes and fibrogenic genes. These findings will confirm whether intrarenal RAS activation will induce ER stress and contribute to RPTC apoptosis in diabetes.

6.1.2 Insulin treatment

Since hyperglycaemia initiates diabetes complications in organs, 10-week-old Akita and Akita-Agt-Tg mice will be implanted with insulin pellets (LinBit, insulin release rate: approximately 0.1 unit/implant/d for >30 d), which will continuously release insulin to normalize blood glucose. We anticipate attenuated kidney pathology and reduced pro-apoptotic and fibrogenic gene

expression. However, the effects of insulin can vary if insulin resistance occurs after the insulin pellets are implanted into the mice, when they have already been diabetic for 5 to 6 weeks. Thus, insulin might need to be administered intraperitoneally at an early age, i.e. 5-10 weeks, with insulin pellet implantation to prevent the development of insulin resistance in Akita mice.

6.1.3 In vitro study to delineate the relationship between intrarenal RAS activation and ER stress

Stable clones overexpressing RAS components (i.e., Agt, AT1R) of IRPTCs will work as in vitro models to explore the effects of hyperglycaemia and intrarenal RAS activation on ER stress induction. In addition, stable clones will be treated with ACE inhibitors and ARBs or insulin to mimic the in vivo condition and ascertain if attenuation will be achieved.

6.1.4 Updated data requiring further experiments on Akita and Akita-rAgt-Tg mice with RAS blockade

Preliminary data from Akita and Akita-rAgt-Tg mice treated with RAS blockers are more convincing than our previous report on STZ-induced diabetic Agt-Tg mice. For example, the albumin/creatinine ratio (ACR) was significantly decreased in Akita and Akita-rAgt-Tg mice treated with RAS blockers, while no obvious improvement in histopathological changes was observed in the treated groups with HE staining. One possible reason for the discrepancy may be the time point when the diabetic mice received treatment. Diabetic Akita mice were given RAS blockers at age 10 weeks. However, Akita mice develop diabetes at age 3-4 weeks; they are diabetic for more than 6 weeks before the treatment begins. Moreover, the treatment lasts for only 6 weeks. Thus, RAS blockers were effective in improving renal function, such as the ACR, but not enough to significantly attenuate the histological abnormalities. In fact, this phenomenon is often seen in clinical practice: It takes less time to achieve functional improvement, but histological amelioration usually requires earlier and longer therapy. In our on-going experiments, we have just begun to treat another group

of diabetic mice with RAS blockers, starting at week 7 instead of week 10. We should have the data sometime between April and May 2009.

6.2 Double Tg mice overexpressing rAgt and CAT in RPTCs

In our laboratory, another strain of Tg mice overexpressing CAT in RPTCs exhibited lowered ROS generation, suppressed intrarenal RAS gene expression, and decreased apoptosis in RPTCs [252, 253]. Therefore, crossbreeding rAgt-Tg mice with CAT-Tg mice can be an ideal model to confirm the effect of both rAgt and CAT on ROS generation and RPTC apoptosis. In brief, homozygous rAgt-Tg mice will be crossbred with homozygous CAT-Tg mice to generate rAgt-CAT heterozygous mice, which will be crossbred again to generate homozygous rAgt-CAT-Tg mice. These mice will then be used for experimentation. All hybrid Tg mice will be identified by Southern blotting of genomic DNA for rAgt and CAT transgene expression. Then, as already described in the above experiments, parameters of apoptosis, fibrogenesis and ER stress induction will be investigated. It is anticipated that rAgt-CAT-Tg mice will exhibit lower ROS generation, decreased pro-apoptotic and fibrogenic gene expression, down-regulated ER stress genes, and improved renal function.

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